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IN THE NEWT IRIS DURING LENS REGENERATION

BY

WILLIAM M. KULYK

C

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Dedication

This thesis is dedicated to my wife, Marilyn, whose support, patience, and self-sacrifice have allowed me the privilege of pursuing my academic interests.

ABSTRACT

Lens regeneration in newts involves the dedifferentiation of pigmented iris epithelial cells and their conversion into lens fibers at the dorsal pupillary margin. The present study describes the patterns of incorporation of radioactive precursors into glycosaminoglycans (GAGs) of the iris during the process of lens regeneration in the newt, *Notophthalmus viridescens*. In addition, this study demonstrates that the newt iris contains an endogenous endoglycosidase that is capable of degrading hyaluronate and chondroitin sulfate polymers.

Adult newts were administered injections of $^{35}\text{SO}_4$ or ^3H -glucosamine at various intervals after lens removal. Iris tissues were excised, pooled, and homogenized. Label incorporated into newly synthesized GAGs was measured from radioactivity accumulated in the cetylpyridinium chloride-precipitable fractions of the tissue homogenates. Compared with the normal iris of the unoperated eye, the uptake of $^{35}\text{SO}_4$ label into sulfated GAGs was elevated two to threefold in the iris after lens removal. Incorporation of $^{35}\text{SO}_4$ into sulfated GAGs rose to a peak at 15 days after lentectomy and declined slightly from its maximum by day 30. The proportions of label in sulfated GAGs of the chondroitin sulfate, dermatan sulfate, and heparin/heparan sulfate types were estimated from the reductions in label

after chondroitinase AC, chondroitinase ABC, and nitrous acid treatments. In the normal iris and at all stages of regeneration, approximately 60% of total $^{35}\text{SO}_4$ was present in heparin/heparan sulfate, while chondroitin sulfate and dermatan sulfate each accounted for 15-25% of label uptake. Labelling of sulfated GAGs was always nearly equivalent in dorsal (lens-forming) and ventral (non-lens-forming) halves of the iris. Uptake of $^{35}\text{SO}_4$ into GAGs was negligible in mature (normal) and developing (30-day regenerate) lenses.

Incorporation of ^3H -glucosamine into hyaluronate was monitored by labelling of cetylpyridinium chloride-precipitable material that was susceptible to *Streptomyces* hyaluronidase digestion. ^3H -glucosamine uptake into hyaluronate was elevated four to seven-fold in the dorsal half of the iris between days 10 and 15 after lens removal, corresponding to the period of most pronounced dedifferentiation in the dorsal iris epithelium. Labelling of hyaluronate was increased comparatively little in the ventral iris after lentectomy.

Cell-free extracts of iris tissue homogenates exhibited hyaluronidase activity capable of liberating N-acetylhexosamine end-groups from polymeric hyaluronate and chondroitin sulfate substrates. The enzyme was identified as an endo-N-acetylhexosaminidase with a pH optimum of 5.0-5.5. Extracts of normal and regenerating irises all displayed high levels of hyaluronidase activity.

The results suggest that the net rates of hyaluronate and sulfated GAG synthesis are dramatically elevated in the iris during lens regeneration. Enhanced sulfated GAG production may be associated with the metabolic activities of iris epithelial cells that undergo only partial dedifferentiation and then redifferentiate into pigment cells. Elevated hyaluronate synthesis in the dorsal iris may be associated with the dedifferentiation of those iris epithelial cells that undergo complete depigmentation and are then converted into lens fibers. The hyaluronidase of the new iris may be involved in hyaluronate and chondroitin sulfate catabolism in the iris epithelium.

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INTRODUCTION

I. Statement of Purpose

Among the most intriguing of biological phenomena is the remarkable ability of some animals to extensively reconstruct bodily parts lost through injury. Within the vertebrates, the capacity for organ regeneration is most highly developed in the urodeles, the newts and salamanders that comprise the order *Caudata* of the class *Amphibia*. Many species of urodeles, as both larvae and adults, can replace fore- and hind-limbs, tails, portions of the brain, lower jaw, intestine, and spinal cord, and various structures of the eye (Hay, 1966; Goss, 1969).

Of particular interest in the study of cell biology is the process by which some urodeles can regenerate the lens of the eye from the tissue of the pigmented iris. In larval and adult newts of the genus *Notophthalmus* (= *Triturus* = *Triton* = *Diemyctylus* = *Taricha*), surgical removal of the lens of the eye triggers an intricate sequence of morphological and biochemical changes in the cells of the adjacent iris tissue. The heavily pigmented cells of the iris epithelium, which are normally mitotically quiescent, are activated into rapid proliferation (T. Yamada and Roesel, 1971). Concomitantly, cytoplasmic depigmentation is accomplished through an active discharge of intracellular

melanin granules (Eguchi, 1963; Karasaki, 1964). The originally compact epithelium of iris cells is disrupted through a reduction of intercellular adhesive contacts (Dumont and Yamada, 1972). These cellular changes are most pronounced at the dorsal pupillary margin of the iris where the cells *dedifferentiate*, or lose the morphological traits characteristic of the intact iris epithelium (T. Yamada, 1976). The proliferating depigmented cells at the margin of the dorsal iris then *redifferentiate* along a new pathway of phenotypic expression. Lens-specific proteins are synthesized, the cells elongate and are converted into lens fibers (Eguchi, 1964; McDevitt and Brahma, 1982). A structurally complete lens is established within 30 to 40 days after lens extirpation. Meanwhile, in the remaining regions of the iris, the partially depigmented iris epithelial cells cease proliferation, resynthesize pigment granules, and reconstitute the original pigment iris epithelium (T. Yamada, 1977).

A central problem in cellular and developmental biology is to uncover the molecular mechanisms which regulate *differentiation*, the process by which cells acquire and stabilize their specialized morphological and functional identities. From this standpoint, the system of lens regeneration in newts affords unique opportunities for investigation. It provides a rare example of phenotypic plasticity among the cells of an adult vertebrate tissue. Following lens removal, fully differentiated cells of the iris

epithelium are released from their embryonically determined pathway of gene expression, a pattern which under normal conditions would remain stable for the lifetime of the organism. Through a process of dedifferentiation, the iris cells abandon their former morphological characteristics and become receptive to a reprogramming of synthetic activities. At the dorsal pupillary margin, progeny of the depigmented iris epithelial cells adopt a radically different phenotype, that of the translucent lens fiber cells. T. Yamada (1977) has emphasized that this capacity for alteration of phenotype, or *cell-type conversion*, runs contrary to the general situation in mature vertebrate tissues wherein differentiation involves an irrevocable commitment to a single pathway of genomic expression. It is possible that knowledge of the molecular events which underlie the processes of dedifferentiation and redifferentiation during newt lens regeneration might clarify common factors involved in maintaining specialized cell structure and function in vertebrate tissues and the manner in which differentiated cell function is disrupted under pathological conditions such as neoplasia.

Classical studies on lens regeneration in the newt suggest that within the intact eye cell-type conversion at the dorsal pupillary margin may be suppressed through the action of an inhibitory factor within the existing lens (for reviews, see Reyer, 1954, 1962). In addition there is evidence that a neuro-humoral factor released from the

retina may promote lens regeneration when the original lens is absent (see Reyer, 1977). Little is known, however, of the biochemical nature of these factors or of the molecular processes which coordinate the intricate morphological changes during iris epithelial cell dedifferentiation and redifferentiation. In the recent past, micro-biochemical approaches to the study of newt lens regeneration have focussed upon nucleic acid metabolism, the synthesis of lens-specific proteins, and the activities of several anabolic and lysosomal enzymes within iris epithelial cells following lens removal (reviewed by T. Yamada, 1977). A new direction of inquiry was suggested by studies which demonstrated that there is a transient decrease in the external surface charge of newt iris epithelial cells during dedifferentiation (Zalik and Scott, 1972). This decrease was at least partially attributable to the disappearance of cell-surface components sensitive to enzymes which remove terminal sialic acid groups and degrade glycosaminoglycans (Zalik and Scott, 1973; Zalik *et al.*, 1976). Evidently, the alterations of iris cell morphology during lens regeneration are accompanied by changes in macromolecular constituents at the cell periphery. These observations may be of considerable significance since in a wide variety of other developmental systems the macromolecular components of the cell surface and the intercellular milieu are believed to play pivotal roles in the mediation of the cell and tissue interactions required for organ formation (Slavkin and

Greulich, 1975; Poste and Nicolson, 1976; Lash and Burger, 1977). Of particular interest is the potential involvement of glycosaminoglycans in the cellular events of lens regeneration. In numerous biological systems a correlation has been established between the progress of cytodifferentiation during embryonic development and modulations in the glycosaminoglycan (GAG) composition of extracellular matrices associated with the tissues (Toole, 1981). Experimental studies *in vitro* have suggested that GAGs might be involved in such diverse phenomena as the suppression of differentiation (Toole, 1973), inhibition of mitosis (Chiarugi and Vannucchi, 1976), stabilization of epithelial morphology (Bernfield *et al.*, 1973), regulation of cellular adhesiveness (Underhill and Dorfman, 1978), and the initiation of inductive tissue interactions (Landstrom and Lovtrup, 1977). In view of the putative role of glycosaminoglycans in the regulation of differentiation and morphogenesis (Toole, 1976) and in light of the paucity of information regarding the molecular changes within the extracellular matrices of the iris during newt lens regeneration, it was felt that an investigation of the possible involvement of glycosaminoglycans in newt lens regeneration would be valuable.

The purpose of this thesis research project has been to examine in detail the dynamics of glycosaminoglycan synthesis and degradation within the newt iris during the morphological changes of dedifferentiation and redifferentia-

tion which lead to lens regeneration. These studies have relied heavily upon techniques of micro-biochemical analysis and radioisotope tracer methodology, and to a lesser extent upon autoradiographic methods.

II. The Phenomenon of Lens Regeneration in Newts

A) *The Study of Lens Regeneration in Historical Perspective:*

The discovery of the phenomenon of lens regeneration has its historical roots in studies conducted during the eighteenth and nineteenth centuries which described the capacity of newts to reconstitute various ocular structures following surgical extirpation of extensive areas of the eye (Bonnet, 1781; Blumenbach, 1787; Philipeaux, 1880; as cited by T. Yamada, 1977). This ocular regeneration was first examined at the microscopic level by Colucci (1891), using histological sections prepared from eyes of adult newts of the species *Triturus cristatus* at several time periods after removal of the outer quarter of the eye. Colucci (1891) observed that following regeneration of the iris itself, the lens was formed from the epithelium at the dorsal margin of the iris. Wolff independently arrived at the same conclusion following a series of detailed histological studies on lens regeneration in larvae and adults of *Triturus taeniatus* and *Triturus cristatus* (Wolff, 1894, 1895, 1901, 1903). Wolff introduced the technique of stimulating lens regeneration in the absence of regeneration of other ocular tissues by removing the lens of the eye through an incision in the cornea. Wolff was also first to report the appearance of phagocytic cells

resembling leukocytes in the vicinity of the iris during the period of lens regeneration and he provided a morphological account of iris epithelial depigmentation. In recognition of these extensive contributions, the phenomenon which he described has been traditionally referred to as "Wolffian lens regeneration."

In the early half of the present century the methodology of the experimental embryologists of the time was extended towards investigation of the lens regeneration phenomenon. Using techniques of tissue transplantation and grafting, attention was focussed on questions of the nature of the initial stimulus for lens regeneration, the distribution of lens-forming capacity in ocular tissues, and the roles of the lens and retina in suppressing and stimulating lens regeneration, respectively (see Reyer, 1954 for a review). The general conclusions arising from these studies will be summarized in a subsequent section of this introduction.

During the latter half of the twentieth century cytochemical, autoradiographic, and micro-biochemical techniques began to be employed in attempts to elucidate the molecular events underlying the overt morphological changes of lens regeneration. This trend of inquiry was most extensively exploited by T. Yamada and his co-workers in investigations of nucleic acid synthesis, enzyme activities, cyclic AMP metabolism and lens protein synthesis during lens regeneration (see T. Yamada, 1967, 1977).

Concomitantly, the development of the electron microscope as a tool of biological analysis allowed for descriptions of the ultrastructural changes within the iris during lens regeneration (Eguchi, 1963, 1964; Karasaki, 1964; Dumont and Yamada, 1972) at a level of detail far exceeding that of earlier histological studies restricted to light microscopy. Finally, in recent years the refinement of techniques for *in vitro* organ and cell culture of newt iris tissue (T. Yamada *et al.*, 1973; Eguchi *et al.*, 1974; Horstman and Zalik, 1974; Cuny and Zalik, 1981) has provided systems for investigating the potential of isolated iris epithelial cells to differentiate as lens fibers and for determining the influence of exogenous agents upon this differentiative capacity.

B) *Taxonomic Distribution of
the Capacity for Lens Regeneration:*

The taxonomic distribution of the capacity for lens regeneration has been surveyed most thoroughly by Stone (1967). At present, 18 species of urodeles have been unequivocally demonstrated to possess the ability to regenerate the lens of the eye from dorsal iris tissue (Table 1). Within the family *Salamandridae*, all of the species of the genus *Notophthalmus* (= *Triturus* = *Triton* = *Taricha* = *Diemictylus*) that have been examined up to this time are able to regenerate the lens as both larvae and adults. In addition, adults and larvae of the species *Salamandra salaman-*

Table 1. Urodeles which have been demonstrated to regenerate lens from dorsal iris.
(adapted from Stone, 1967)

family Salamandridae*

Notophthalmus viridescens (including subspecies

N. v. dorsalis,
N. v. louisianensis,
N. v. peristriatus, and
N. v. viridescens)

Taricha granulosa
Triturus alpestris
Triturus cristatus
Triturus eniscauda
Triturus helveticus
Triturus marmoratus
Triturus pyrrhogaster
Triturus rivularis
Triturus sierra
Triturus taeniatus
Triturus torosus

Pleurodeles waltii
Salamandrina perspicillata
Salamandra salamandra salamandra

family Plethodontidae

Eurycea lucifuga (larvae only)
Tryphlotriton spelaeus (larvae only)

*Within the family Salamandridae, the generic names *Notophthalmus*, *Taricha*, *Triturus*, *Triton*, and *Diemictylus* are taxonomically equivalent.

dra salamandra (=maculosa) and *Salamandrina perspicillata* and adults of the species *Pleurodeles waltii* are known to possess this capacity. Within the family *Plethodontidae*, the cave-dwelling species *Tryphlotriton spelaeus* and *Eurycea lucifuga* have been demonstrated to regenerate lenses in the larval stage.

In the majority of these species the site of new lens formation is the mid-dorsal margin of the iris. However in *Salamandra s. salamandra* the lens differentiates at the temporo-dorsal margin of the iris and in the larvae of *Eurycea lucifuga* (and some *Tryphlotriton spelaeus* larvae) the regenerated lens is derived from cells on the inner border of the dorsal iris, a short distance from the margin (Stone, 1967).

It is important to note that the list of urodelan species known to possess the capacity for lens regeneration is by no means necessarily complete. Many species of newts and salamanders have yet to be critically evaluated with respect to their competence for lens regeneration. A number of species which have been examined adequately and demonstrably lack the ability to regenerate lens from iris tissue are listed in Table 2.

As a postscript to this discussion it is interesting that outside of the urodeles of the class *Caudata*, the capacity for lens regeneration from iris tissue has been substantiated in only one other vertebrate group, a species of Japanese fresh water fish, *Misgurnus anguillicaudatus*,

Table 2. Some urodeles which have been demonstrated to lack the capacity for lens regeneration.
(adapted from Stone, 1967)

family *Ambystomidae*

<i>Ambystoma mexicanum</i>	(larvae)
<i>Ambystoma opacum gravis</i>	(larvae)
<i>Ambystoma punctatum</i>	(larvae)
<i>Ambystoma tigrinum</i>	(larvae)

family *Plethodontidae*

<i>Batrachoceps attenuatus attenuatus</i>	(adults)
<i>Desmognathus fuscus auriculatis</i>	(adults)
<i>Ensatina eschscholtzii picta</i>	(adults)
<i>Eurycea longicauda</i>	(adults)

belonging to the family *Cobitidae* (Sato, 1961; cited by Stone, 1967).

A substantially different phenomenon is the capacity for regeneration of lens from ocular tissues other than the iris. Tadpoles of the anuran *Xenopus laevis* as well as young larvae of the urodelan *Hynobius unangso* have been demonstrated to regenerate lens tissue from the outer epithelium of the cornea following lentectomy (Freeman, 1963; Reyer, 1977). Furthermore, in many vertebrate species including mammals, lens tissue can regenerate to some extent from fragments of lens epithelium remaining in the optic chamber after incomplete lens removal. However, these forms of lens regeneration do not represent systems in which fully differentiated adult tissues undergo a conversion of cell-type in a manner comparable to Wolffian lens regeneration. Both the corneal epithelium and the lens epithelium remain as mitotically active stem cell populations throughout the life of the animal and as such cannot be considered as structurally specialized, fully differentiated cell-types.

C) Anatomy of the Newt Eye:

As a preface to a description of the morphological events during lens regeneration, it is worthwhile to review briefly the gross anatomy and histological architecture of the eye of the newt. This outline relies heavily upon a description of the structure of the eye of *Notophthalmus*

viridescens as supplied by Reyer (1977); with additional information obtained from the general textbooks of ocular histology by Hogan *et al.* (1971) and Fine and Yanoff (1979).

A median section through the eye of the adult newt, *Notophthalmus viridescens*, is illustrated in Figure 1. The outer surface of the eye is delimited anteriorly by the cornea and posteriorly by the connective tissue sclera. The cornea is transparent *in vivo* and serves as the first site for refraction and transmission of incident light. The external surface of the cornea is covered by a cuboidal epithelium several cell layers in thickness. Beneath the corneal epithelium lies an amorphous basement membrane which is homologous to, but thinner than, the Bowman's layer of the human eye. Beneath this layer lies a thick connective tissue stroma composed of numerous lamellae of parallel collagen bundles embedded within an amorphous ground substance. The inner surface of the cornea lacks a conspicuous endothelial cell layer. The collagenous sclera, together with the cornea, forms a tough fibrous tunic around the eye.

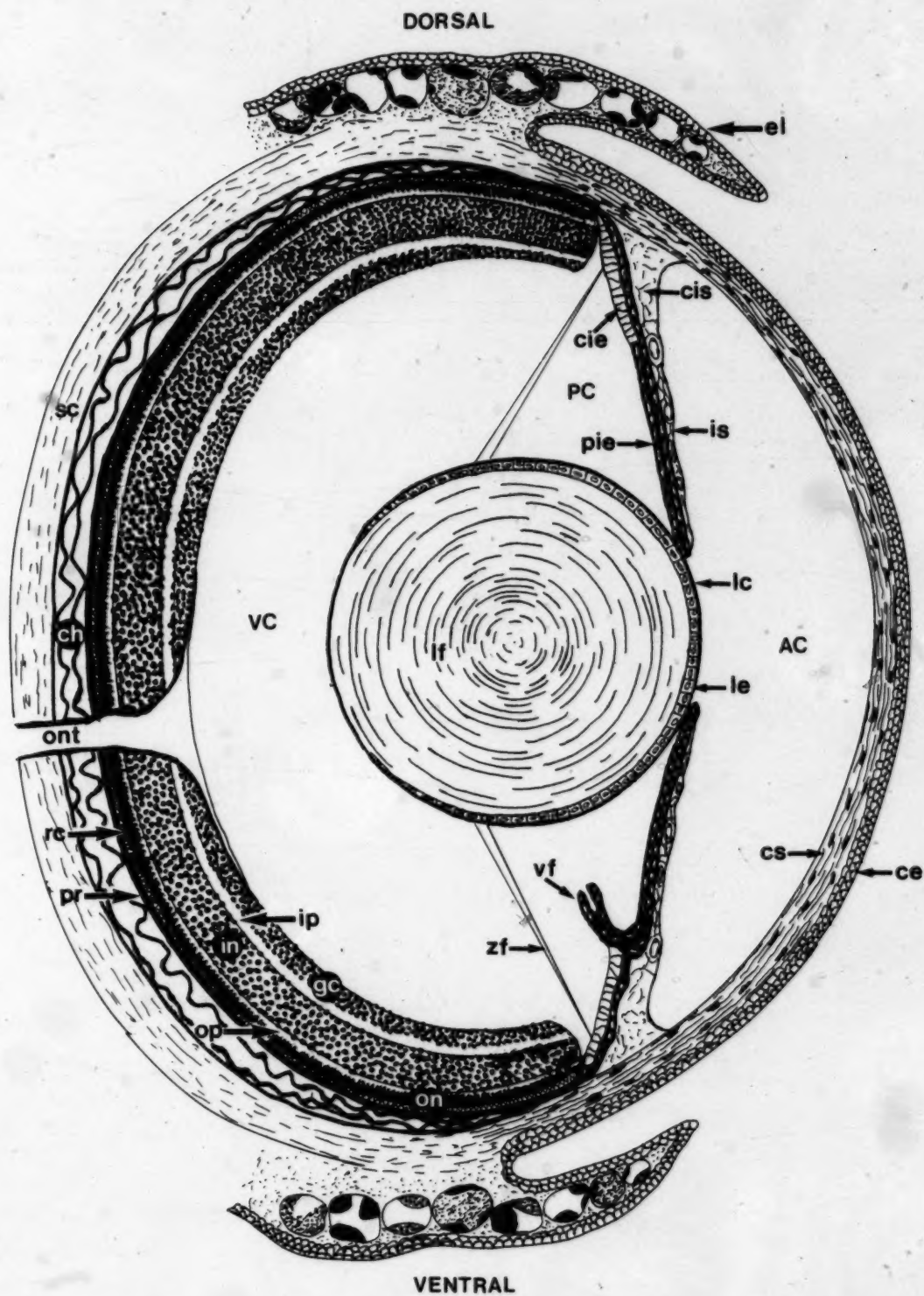
Just interior to the sclera lies the vascular or uveal tunic of the eye. Posteriorly, the choroid appears as a network of heavily pigmented lamellae. Numerous vessels in this region constitute the major vascular blood supply of the eye. More anteriorly, the stroma of the ciliary body contains numerous vessels which function in the production and drainage of the aqueous humour in the anterior chamber.

Figure 1.

Schematic illustration of the eye of the newt, *Notophthalmus viridescens*, as seen in a median sagittal section.

Abbreviations:

AC	= anterior chamber of the eye
ce	= corneal epithelium
ch	= choroid
cie	= ciliary epithelium
cis	= stroma of ciliary body
cs	= corneal stroma
el	= eyelid
gc	= ganglion cell layer of neural retina
in	= inner nuclear layer of neural retina
ip	= inner plexiform layer of neural retina
is	= iris stroma
lc	= lens capsule
le	= lens epithelium
lf	= lens fibers
on	= outer nuclear layer of neural retina
ont	= optic nerve tract
op	= outer plexiform layer of neural retina
PC	= posterior chamber of the eye
pie	= pigmented iris epithelium
pr	= pigmented retinal epithelium
rc	= layer of rods and cones of the neural retina
sc	= sclera
VC	= vitreous chamber of the eye
vf	= ventral fold of ciliary body
zf	= zonula fibers



of the eye. Ventrally, the ciliary body is extended in a single medial fold, or process, containing the *protractor lentis* muscle which controls the accommodation mechanism of the eye. This mid-ventral fold is a vestige of the choroid or fetal fissure of embryonic development.

The internal tunic of the eye consists of the pigmented and neural retinas posteriorly and the ciliary epithelium and iris epithelium anteriorly. The *neural retina* is comprised of the layers characteristic of vertebrate eyes. From anterior to posterior these are: the ganglion cell layer, the inner plexiform layer, the inner nuclear layer, the outer plexiform layer, the outer nuclear layer, and the region of rods and cones. The *pigmented retinal epithelium* lies just posterior to the rod and cone layer of the neural retina and extends thin processes around the outer portions of the rods and cones. Cells of the pigmented retina owe their dense pigmentation to a high concentration of intracellular melanosomes.

The *iris* is composed of a two-layered *pigmented iris epithelium* and an overlying connective tissue *stroma*. The iris stroma is a loose connective tissue containing numerous endothelium-lined capillary vessels which supply blood to the underlying pigmented iris cells. The stroma is also perforated by many lacunae or fenestrations which allow the aqueous humour in the anterior chamber of the eye to bathe the underlying iris epithelium. A variety of cell-types are sparsely distributed within the iris stroma, including

melanophores, iridophores, fibroblasts, and mast cells. Pupillary constriction and dilation are controlled by circumferentially-oriented sphinctor and radially-oriented dilator smooth muscle bundles within the stroma (Reyer, 1977). The iris epithelium is composed of two layers of heavily pigmented cuboidal cells. In both the inner and outer laminae the iris epithelial cells are densely packed with melanosomes, making details of cellular structure difficult to discern by conventional light microscopy. Using electron microscopy, it has been shown that the inner surface of the inner epithelial lamina as well as the outer surface of the outer lamina are invested by thin extracellular basement membranes (Dumont and Yamada, 1972; Fine and Yanoff, 1979). Laterally, the boundary of the iris epithelium is marked by a sharp transition from the pigmented cells of the inner iris epithelial lamina to the non-pigmented cells of the inner layer of *ciliary epithelium*. The outer layer of ciliary epithelium is pigmented.

The lens of the eye is very large and occupies much of the ocular chamber posterior to the iris. The urodele lens is nearly spherical in shape. Anteriorly the lens is covered by a single cell layer of *lens epithelium*. The lens epithelium disappears posteriorly at the equatorial margin of the lens. The bulk of lens tissue is composed of elongated lens fiber cells which are tightly packed in a concentric arrangement around a central core, or nucleus of the lens. Lens fibers in all but the most superficial

regions lack nuclei and are virtually devoid of intracellular organelles. The fibers have a high content of specialized proteins, the α , β , and γ crystallins, which impart the transparent optical properties to the lens. The entire outer surface of the lens is invested by an amorphous basement membrane, the *lens capsule*. The lens is held in place within the eye by a circumferential corona of suspensory ligaments, or *zonula fibers*, which extend from the ciliary body to the equatorial region of the lens capsule. (The zonula fibers are not preserved well under standard histological procedures). Accommodation, or focussing of the lens, is accomplished by contraction and relaxation of the single *protractor lentis* muscle located in the mid-ventral fold of the ciliary stroma. Contraction of this muscle applies tension on the zonula fibers, thereby pushing the lens forward in the ocular chamber.

The various structures of the eye subdivide the ocular chamber into three distinct regions. The *anterior chamber* occupies the space between the inner surface of the cornea and the outer surface of the iridal stroma and lens. The *posterior chamber* denotes the region posterior to the inner surface of the iris and anterior to the corona of zonular fibers. The *vitreal chamber* constitutes the area posterior to the zonula fiber complex and lens and anterior to the neural retina. In the newt the vitreal body is a delicate fluid-filled sac of amorphous material, unlike the highly collagenous vitreal body of the mammalian eye.

D) *Sequence of Morphological Changes within
the Iris During Lens Regeneration:*

Although the morphological events which culminate in the regeneration of a lens from the dorsal iris following lentectomy proceed in an ordered temporal sequence, the absolute rate of lens regeneration is directly proportional to the ambient temperature, owing to the poikilothermic metabolism of the urodeles. Regeneration is greatly retarded at temperatures below 12°C and is most rapid at temperatures from 26°C up to the limit of viability of the animals (approximately 34°C) (Nakamura, 1935; Donaldson and Chan, 1973). Rates of lens regeneration differ in various species of newts (Stone, 1967) and tend to be more rapid in larval than adult animals (Reyer, 1954). In addition, there is considerable individual variation in the rates of lens regeneration among newts of the same species and of equivalent developmental age (Yamada, 1967; Connelly, 1978). Despite the many factors that influence the absolute rate of lens regeneration, a system was required by which the process of lens regeneration could be compared in different species and in different laboratories. For this purpose, Sato (1940) devised a scheme of morphological stages representing the temporal sequence of changes within the iris following lens removal as revealed through light microscopic examination of median sagittal sections through the eyes of adult *Triturus pyrrhogaster* and *Triturus taeniatus*. The Sato staging system has since been adapted to

the time course of lens regeneration in adults and larvae of several other urodele species. This staging system, as modified by Yamada (1967) for adults of *Notophthalmus viridescens* (Figure 2), will be described below as it provides a useful introduction to the cellular and subcellular events which characterize the process of lens regeneration. Furthermore, it relates these morphological changes to the average time course of lens regeneration as expressed in days after lentectomy. Additional information pertaining to the ultrastructural changes of lens regeneration as revealed through studies with the electron microscope (Eguchi 1963, 1964; Karasaki, 1964; Dumont and Yamada 1972; T. Yamada and Dumont, 1972) have been included in the following description.

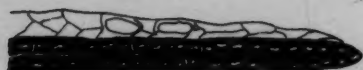
Normal iris:

The normal iris of the intact eye is composed of an inner lamina (facing the posterior, retinal region of the eye) and an outer lamina (facing the cornea) of heavily pigmented epithelial cells. Each epithelial lamina is a single cell layer in thickness. The adjacent cells within each lamina are in close contact and feature numerous desmosome-type intercellular junctions (Karasaki, 1964; Dumont and Yamada, 1972). The inner and outer epithelial lamina are tightly apposed, with the cells of the two laminae being bound by specialized intercellular junctions (Karasaki, 1964). The most conspicuous morphological

Figure 2.

Schematic illustrations of the sequence of morphological changes in the dorsal iris during lens regeneration in the adult newt, *Notophthalmus viridescens*. (Adapted from drawings by T. Yamada, 1967.) Each diagram represents the tip of the dorsal iris as seen in a median sagittal section through the eye. The iris epithelium and the overlying iris stroma are illustrated. Pigmented epithelial cells are portrayed as black; partially depigmented cells are stippled; fully depigmented cells of the lens regenerate are unshaded. Invading macrophages and mast cells are not shown.

Roman numerals correspond to the morphological stages of lens regeneration according to the scheme of Sato (1940) as applied to *N. viridescens* by T. Yamada (1967). Arabic numerals refer to the time course of lens regeneration expressed in days after lentectomy, as observed by T. Yamada (1967) for adult *N. viridescens* reared at $21 \pm 1^\circ\text{C}$.



Normal Iris



Stage I (3-6 days)



Stage II (6-10 days)



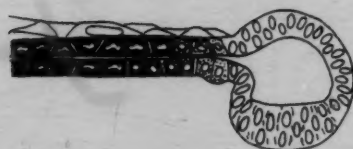
Stage III (8-11 days)



Stage IV (9-15 days)



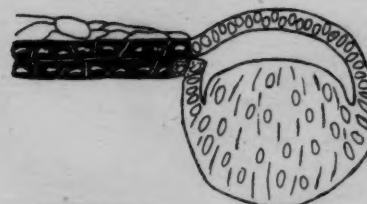
Stage V (12-15 days)



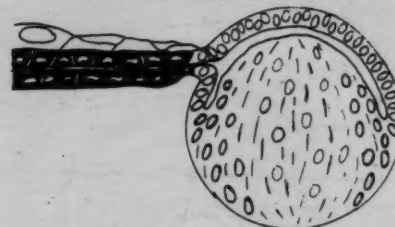
Stage VI (12-16 days)



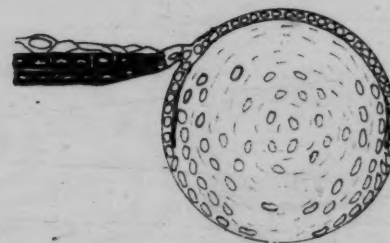
Stage VII (15-18 days)



Stage VIII (15-19 days)



Stage IX (18-20 days)



Stage X (18-25 days)



Stage XI (21-30 days)

feature of the normal iris epithelial cells is a dense accumulation of pigment in the form of membrane-bound melanin granules which are randomly distributed through the cytoplasm (Eguchi, 1963; Karasaki, 1964). The iris epithelial cell nuclei have irregular contours owing to numerous indentations in the nuclear envelope. Within each nucleus lies one or more small, dense nucleoli. The nuclei are surrounded by a large volume of cytoplasm in which vesicular structures of the endoplasmic reticulum and Golgi apparatus are difficult to distinguish (Eguchi, 1963; Dumont and Yamada, 1972). Ribosomes are sparsely distributed in small clusters lying freely within the cytosol (Karasaki, 1964). Mitochondria are also few in number and tend to be concentrated at the free boundaries of the iris cells (Karasaki, 1964) and are reportedly more abundant in cells at the dorsal margin of the iris than in those at the ventral margin (Eguchi, 1963). In all other respects the fine structure of the dorsal iris (from which lens tissue can regenerate) does not differ conspicuously from that of the lateral and ventral regions (which do not form lens tissue). Extracellularly, the posterior surface of the inner epithelial lamina and the anterior surface of the outer lamina are covered by a thin, amorphous basement membrane (Karasaki, 1964; Dumont and Yamada, 1972). Overlying the basement membrane of the outer iridal lamina is a thick stroma of loose connective tissue containing small blood vessels and a heterogeneous population of fibro-

blasts, iridophores, melanophores, and mast cells.

Stage I (3-6 days after lentectomy):

In the region of the pupillary margin, the iris epithelium thickens due to both a relaxation of the iris around the pupil (Eguchi, 1963) and a slight elongation of iris epithelial cells along their apico-basal axes (Dumont and Yamada, 1972). A space appears between the two layers of the iris epithelium as a result of reduced intercellular adhesion between opposing surfaces of neighbouring cells in the inner and outer iridal laminae (Karasaki, 1964). At the same time intercellular spaces are enlarged between the lateral surface of adjacent cells within each iris lamina (Dumont and Yamada, 1972). At the dorsal pupillary margin the nuclei of the iris cells begin to swell and gradually lose their indentations becoming more spherical or ellipsoid in shape (Karasaki, 1964). Within each nucleus, the nucleoli increase in number but retain a condensed appearance as in the normal iris. Ameboid macrophages of monocytic origin begin to invade the iris epithelium from the stromal circulatory system (Eguchi, 1963; T. Yamada and Dumont, 1972) along with mast cells (Setoguti *et al.*, 1963) and a heterogeneous population of granulocytes (T. Yamada and Dumont, 1972). The condition of the iris at this stage resembles a state of "inflammation" (Eguchi, 1963) and is not markedly different in dorsal and ventral regions of the tissue. Unlike the normal iris, which is mitotically

quiescent, occasional mitotic figures appear in the pigmented epithelial cells surrounding the margin of the iris by four days after lens removal (T. Yamada and Roesel, 1971).

Stage II (6-10 days after lensectomy):

The interlaminar space expands and the iris thickens further due to elongation of pigmented epithelial cells (Dumont and Yamada, 1972). Intracellular melanosomes move to the periphery of the iris epithelial cells (Eguchi, 1963) and are released from pseudopod-like cytoplasmic processes at the basal ends of the cells (Dumont and Yamada, 1972). At the pupillary margin, the numbers of intracellular melanosomes are reduced as pigment granules are extruded either singly or in groups in exocytotic membrane-bound vesicles (Dumont and Yamada, 1972; Eguchi, 1963). The discharged melanosomes are engulfed by pseudopodial extensions of macrophages which have invaded the iris laminae from all surfaces and frequently enter the interlaminar space (Eguchi, 1963; Karasaki, 1964; T. Yamada and Dumont, 1972). The basement membrane of the iris epithelium is disrupted at locations where several macrophages have simultaneously penetrated it (T. Yamada and Dumont, 1972). Concomitant with the depigmentation activities of the iris epithelial cells, proliferative activity is high in these cells as indicated by frequent mitotic figures (T. Yamada and Roesel, 1971). The interphase

nuclei continue to swell and adopt a rounded configuration. Nucleoli have increased in number and have enlarged owing to the acquisition of a granular cortical region, apparently composed of ribosomal precursor particles (Karasaki, 1964). Vesicular structures of the rough and smooth endoplasmic reticula and Golgi apparatus become more prominent in the cytoplasm, especially in the region around the nucleus (Eguchi, 1963; Dumont and Yamada, 1972). The population of free ribosomes, frequently in polysome clusters, increases as does the number of mitochondria. Lysosomes are visible in the cytoplasm (Eguchi, 1963) and the majority of iris cells acquire a positive reaction for lysosomal acid phosphatase in the vicinity of intracellular melanosome clusters (T. Yamada *et al.*, 1978).

Stage III (8-11 days after lentectomy):

The continued discharge and autophagy of melanin granules by iris cells (Dumont and Yamada, 1972; T. Yamada *et al.*, 1978) and the persistent activity of macrophages in removing the extruded melanosomes (T. Yamada and Dumont, 1972) have resulted in the complete depigmentation of some cells at the dorsal margin of the iris, especially within the inner iridal lamina. Mitotic activity is high in this region (T. Yamada and Roesel, 1971). Depigmentation and proliferation of iris cells is also evident, albeit to a lesser degree, at lateral and ventral margins of the iris. Many macrophages are densely packed with engulfed melano-

somes. Within the depigmenting iris cells the nuclei remain large and the nucleoli retain a granular cortex indicative of synthetic activity. Free and membrane-bound ribosomes become more conspicuous in the cytoplasm.

Stage IV (9-15 days after lentectomy):

The complete depigmentation of cells within the inner and outer laminae of iris epithelium at the dorsal pupillary margin and an enlargement of the interlaminar space have resulted in the formation of a hollow "lens vesicle." The anterior and posterior walls of the lens vesicle are continuous with the inner and outer pigmented iris laminae, respectively. The cavity of the lens vesicle is continuous with the interlaminar space of the adjacent pigmented region of the iris. Proliferation and depigmentation continue in other parts of the iris but only rarely form vesicular depigmented structures as at the dorsal pupillary margin. Within the cells of the lens vesicle, nuclei remain enlarged while the surrounding cytoplasm diminishes in volume (Dumont and Yamada, 1972). The latter is at least partially attributable to a loss of cytoplasm accompanying the exocytotic discharge of melanosomes in earlier stages (Dumont and Yamada, 1972). Within the lens vesicle there is a high level of proliferative activity.

Stage V (12-15 days after lentectomy):

The lens vesicle has enlarged through rapid prolifera-

tion of the depigmented cell population and the incorporation of newly depigmented cells supplied from the iris laminae (Eguchi, 1964). The anterior wall develops as an epithelium of cuboidal cells in which mitotic activity is vigorous. In the posterior wall, elongation of depigmented cells produces a columnar epithelium. At the boundary of the depigmented and pigmented cells the posterior wall of the lens vesicle is thin and forms a prominent constriction which delimits the lens rudiment from the remainder of the iris (Eguchi, 1964; Connelly, 1978). Within the columnar cells of the posterior wall, nuclei remain large and typically contain several nucleoli with granular cortical regions. Rough endoplasmic reticulum abounds in the cytoplasm (Eguchi, 1964). The density of free ribosomes and polyribosome clusters increases markedly (Eguchi, 1964) imparting a strong cytoplasmic basophilia to these cells (T. Yamada, 1967). In the anterior wall of the lens vesicle the mitochondria are small and globular, but in the posterior wall the mitochondria are large and elaborately branched (Eguchi, 1964). The population of macrophages is reduced in the area of the fully depigmented lens vesicle cells, although macrophages can sometimes be found within the cavity of the lens vesicle. In regions of the iris other than the lens vesicle, proliferation, depigmentation, and macrophage activities remain high.

Stage VI (12-16 days after lentectomy):

Within the growing lens vesicle, the central region of the posterior wall is thickened by elongation of the columnar cells. Nuclei of these cells are long and ellipsoid. Nucleoli remain prominent but some have lost their granular cortex (Eguchi, 1964). Rough and smooth endoplasmic reticular structures are conspicuous in perinuclear and basal regions of the cell. Within the vesicles of the rough endoplasmic reticulum and within the cytosol fine filamentous material starts to accumulate indicating deposition of lens proteins (Eguchi, 1964). Long, intricately branched mitochondria are arranged along the elongation axes of the cells. Mitotic activity ceases within the thickened region of the posterior wall but mitotic figures are numerous in all other regions of the depigmented lens vesicle. Proliferation and depigmentation continue in lateral and ventral regions of the iris.

Stage VII (15-18 days after lentectomy):

The elongating cells in the posterior wall of the lens vesicle have formed a hemispherical process, the *lens fiber hillock*, which projects into the cavity of the lens vesicle. The elongated cells within the fiber hillock mature into the *primary lens fiber* cells. Within these cells, nuclei are elongate and the nucleoli are small and lack a granular cortex (Eguchi, 1964). Towards the centre of this fiber complex, intracellular organelles such as the mito-

chondria and endoplasmic reticulum undergo degeneration. The cytoplasmic basophilia characteristic of earlier stages in the differentiation of these cells is lost as the number of free ribosomes declines (T. Yamada, 1967). The primary lens fibers are tightly packed and in close contact with one another. Fine filamentous material accumulates in the cytosol. In the anterior wall of the lens rudiment, the cuboidal cells maintain a high level of mitotic activity. In the remainder of the iris, the proliferation and depigmentation of iris epithelial cells is greatly reduced.

Stage VIII (15-19 days after lentectomy):

Growth of the lens fiber complex has reduced the cavity of the lens vesicle to a narrow slit beneath the anterior wall of depigmented cuboidal cells. Within the core of the lens fiber complex, the boundaries of individual lens fibers have become obscure and nuclei are less distinct and show signs of degeneration (Eguchi, 1964). Within the cytoplasm the endoplasmic reticulum is no longer visible and other organelles appear in various stages of disintegration. The cytoplasm is filled with fine filamentous and granular materials. At the equatorial region of the lens vesicle, between the primary lens fiber core and the cuboidal cells of the anterior wall, a population of cells ceases to proliferate and elongates in such a manner as to encircle the core of primary lens fibers. These represent the first generation of secondary lens

fiber cells. A thin amorphous basement membrane forms around the entire outer surface of the lens vesicle.

Stage IX (18-20 days after lentectomy):

The anterior wall of the lens rudiment together with the marginal region of the posterior wall forms a flattened lens epithelium over the lens fiber complex. The fiber complex continues to grow by the addition of newly elongating secondary lens fiber cells from the periphery of the posterior wall. Mitotic activity remains high in the anterior lens epithelium. The lens rudiment retains its connection to the dorsal iris through a narrowing stalk of depigmented cells. Within the non-lens-forming regions of the iris proliferative activities cease and the partially depigmented cells begin to resynthesize melanosomes.

Stage X (18-25 days after lentectomy):

The anterior lens epithelium becomes a flattened single cell layer as the cavity of the lens vesicle is obliterated by the growth of the underlying fiber complex. The addition of successive layers of elongated secondary lens fiber cells around the primary lens fiber core has produced a concentric arrangement of lens fiber lamellae in the major portion of the lens rudiment. Maturation of the lens fibers spreads centrifugally from the primary lens fiber core to the deeper layers of secondary lens fibers as evidenced by nuclear and organellar disintegration and

accumulation of filamentous materials of the lens proteins.

Stage XI (21-30 days after lentectomy):

The lens regenerate has acquired the general structure of the original lens but continues to grow as the result of proliferation in the lens epithelium and differentiation of lens fiber cells from elongating cells at the equatorial region of the lens. The stalk disappears and the newly formed lens becomes an autonomously growing organ. Within two or more months the new lens will attain the size of the original structure.

E) *Biochemical Events during Lens Regeneration:*

Processes of morphological change during development are now recognized as the overt manifestation of cumulative changes at the molecular level in the synthetic activities of cells. Differentiation, in turn, entails the fixation of a particular limited pattern of macromolecular synthesis upon a given cell or cell population resulting in the acquisition of specialized structure and function. It is apparent, therefore, that an understanding of the mechanics of morphological change in tissues during embryonic development and organ regeneration must ultimately rely upon an elucidation of the temporal sequence of synthetic activities of the participating cells.

During the last twenty years this philosophy of inquiry was introduced to the study of the newt lens regener-

ation phenomenon by T. Yamada and his co-workers, who investigated the metabolism of a variety of macromolecules within the newt iris following lens removal. These studies have been the subject of a comprehensive review (T. Yamada, 1977) and will be briefly summarized below.

Enhancement of RNA synthesis:

An elevation in the rate of incorporation of radioactive precursors into ribonucleic acid (RNA) is among the earliest of biochemical events detectable within the newt iris epithelium following lentectomy and, indeed, precedes the overt morphological events of iris cell depigmentation and mitosis. Within two days after lens removal enhanced uptake of radioactively labelled uridine into pigmented epithelial cells at the iris margin can be demonstrated by autoradiography (T. Yamada and Karasaki, 1963) or by isolation and fractionation of RNA (Reese *et al.*, 1969; Thorpe *et al.*, 1974). The rate of labelling at the dorsal pupillary margin rises for the first several days after lentectomy, but declines somewhat just prior to lens formation (T. Yamada and Karasaki, 1963). Initially the vast majority of labelled uridine is associated with the nuclei of pigmented cells, but the proportion of label appearing in the cytoplasm gradually increases during subsequent depigmentation and lens vesicle formation (T. Yamada and Karasaki, 1963). Isolation and fractionation of the RNA species within the regenerating newt iris reveals that the

enhanced uptake of labelled uridine following lensectomy is primarily attributable to augmented production and accumulation of 18S and 28S ribosomal RNA subunits, apparently derived by cleavage of a 40S precursor rRNA synthesized within the nucleus (Reese *et al.*, 1969). The four-fold increase in rRNA production during the initial phase of lens regeneration results from both an increased rate of transcription of redundant DNA sequences coding for the rRNA precursor molecule as well as from a 60% amplification in the number of such DNA sequences (Collins, 1972, 1974a). The stimulation of rRNA synthesis within the iris epithelial cells provides the biochemical basis for the observed ultrastructural changes with respect to size, number, and structural complexity of iris cell nucleoli during lens regeneration (Dumont *et al.*, 1970) and for the increase in RNAase-sensitive basophilia within the cytoplasm of depigmenting cells at the iris margin (Takata, 1952; Ogawa, 1962).

Activation of DNA synthesis:

The pigmented epithelial cells of the normal iris of the adult newt are completely quiescent with respect to both DNA synthesis and mitotic proliferation. While corneal epithelial cells and some iris stroma cells in the untraumatized eye incorporate ^3H -thymidine as demonstrated through autoradiography (T. Yamada and Roesel, 1969), neither labelled nuclei nor mitotic figures are ever found

among the pigmented iris epithelial cell population (Eisenberg and Yamada, 1966; T. Yamada and Roesel, 1969). Evidently not even a low level of cell replication is required for maintenance of the cell population of the iris epithelium under normal conditions.

Conversely, following lentectomy the pigmented iris epithelial cells are triggered to initiate DNA replication. Uptake of ^3H -thymidine into some pigmented iris cell nuclei is first apparent by autoradiography at four days after lens removal. By day 5, about 20% of the epithelial cells are engaged in DNA synthesis in both dorsal and ventral iris sectors (Eisenberg and Yamada, 1966; T. Yamada and Roesel, 1969), with three-quarters of the labelled cells appearing in the inner epithelial lamina. It is noteworthy that the onset of DNA synthesis precedes visible signs of depigmentation in these cells.

In subsequent stages a dorso-ventral gradient is established with respect to DNA replicative activities. By day 11 nearly all depigmented cells of the lens vesicle forming at the pupillary margin are labelled. With further growth of the lens rudiment, DNA synthetic activities remain high in the outer wall of the vesicle while elongating cells of the expanding lens fiber complex are no longer labelled (Eisenberg and Yamada, 1966). In the ventral half of the iris the proportion of cells engaged in DNA replication (initially as high as in the dorsal iris) declines gradually after day 7 and becomes negligible by day 30.

(Eisenberg and Yamada, 1966).

Structural changes in the chromosomal DNA of the pigmented iris epithelial cells precede their DNA replicative activities. As revealed by patterns of nitrocellulose binding affinity, thermal denaturation, hydroxyapatite chromatography, and isopycnic density gradient centrifugation, the "native" chromosomal DNA is apparently primed for replicative activity by the introduction of small "nicks" and larger "gaps" in the nucleotide sequence prior to entrance of the pigmented iris cell into the replicative, or S phase, of the cell cycle at day 5 (Collins, 1974a). Using an *in vitro* DNA polymerase system, the modified DNA at this pre-replicative stage has been shown to act as a more effective template for DNA synthesis than the DNA of either the normal iris or of the iris at three days after lentectomy (Collins, 1974b).

Not surprisingly, the onset of DNA synthesis at four to five days after lentectomy is followed by a wave of mitotic activity within the iris epithelial cell population. Mitotic figures are first observable within the pigmented iris at days 4 and 5, and their numbers rise rapidly in both dorsal and ventral iris sectors to a peak at day 7 (T. Yamada and Roesel, 1971). In subsequent stages a dorso-ventral gradient of mitotic activity is established. In the dorsal iris, the proportion of mitotic cells declines slightly by day 10 before rising to a second, more prominent peak at day 15 (T. Yamada and

Roesel, 1971). By day 20 mitotic activity among the pigmented cells of the dorsal iris proper declines again to the low level observed at day 5, while within the regenerating lens rudiment the number of mitotic cells remains high. By day 30 mitotic activity remains only within the lens epithelium of the regenerated lens (T. Yamada and Roesel, 1971). Within the ventral iris the numbers of mitotic cells are as high as in the dorsal iris until day 7, but remain lower than in the dorsal iris at all subsequent stages (Eisenberg and Yamada, 1966; T. Yamada and Roesel, 1971).

The fact that the first wave of mitotic proliferation following lentectomy is preceded by a pulse of DNA synthetic activity indicates that the normal pigmented iris cells are fixed in the G_1 rather than the G_2 phase of the cell cycle. Yamada has designated this arrested state of the G_1 phase as G_0 (T. Yamada, 1977). Lentectomy triggers a transition of the normal pigmented iris epithelial cells from the stationary G_0 phase to the active growth phase of G_1 . Subsequently, DNA synthetic activity is initiated during the S phase of the cell cycle, which is followed by mitotic cell division (M) and a period of post-mitotic growth (G_2).

Protein synthesis:

The function of ribosomal RNA in the machinery of protein synthesis is now an established doctrine of intracel-

lular metabolism. The activation of rRNA production in the iris epithelial cells immediately after lentectomy (see above) might be expected to be preparatory to an augmented elaboration of protein by the cells. Autoradiography of iris tissue labelled with ^3H -leucine at various stages of lens regeneration confirms that there is a large increase in the level of protein synthesis within the iris epithelial cells following lens removal (T. Yamada and Takata, 1963). From days 2 to 8 there is a progressive eight-fold increase in the rate of incorporation of label relative to the non-lentectomized iris epithelium. During this phase of synthetic activity labelling is nearly equivalent in both dorsal and ventral regions of the iris. At the dorsal iris margin this heightened level of leucine incorporation is maintained without appreciable change until day 16. Subsequently, within the region of the depigmented lens rudiment there is a second wave of accelerated labelling. By day 25, uptake of labelled leucine into the lens epithelium of the lens regenerate is elevated forty-fold in comparison with the pigmented epithelium of the normal iris, while labelling is ten-fold greater than normal within the region of the elongated lens fibers (T. Yamada and Takata, 1963). In the ventral iris epithelium there is no corresponding second wave of increased protein synthesis. At the thickened margin of the ventral iris, where some cells undergo depigmentation, protein synthesis is maintained at the level attained at day 8 until at least

day 25.

The function of the first period of enhanced protein synthesis, which occurs throughout the iris during the first eight days following lentectomy, is not yet established. The second wave of synthetic activity localized within the lens regenerate is almost certainly related to the production of the lens-specific proteins, the α , β , and γ crystallins (McDevitt and Brahma, 1982).

Cyclic AMP metabolism:

Changing intracellular levels of adenosine 3',5' cyclic monophosphate (cyclic AMP) are known to mediate the response of eukaryotic cells to hormones, to regulate enzyme activities, and to affect numerous other intracellular functions. A sharp decline in the concentration of cyclic AMP within epithelial cells of the dorsal iris occurs during the first two days after lentectomy, corresponding temporally with the onset of enhanced rRNA synthesis (Thorpe *et al.*, 1974; Velazquez and Ortiz, 1980). Just prior to the onset of DNA synthesis at day 4, the level of intracellular cyclic AMP rebounds rapidly (Thorpe *et al.*, 1974; Velazquez and Ortiz, 1980) and, according to Velazquez and Ortiz (1980), rises to a concentration higher than that of the normal iris, with a heightened level of cyclic AMP being maintained until day 8. The period of elevated cyclic AMP corresponds roughly to the timing of most active depigmentation at the pupillary margin. In *in vitro*

culture of iris epithelial cells, exogenous adenosine, cyclic AMP, and related compounds promote a contraction of peripheral cytoplasm frequently resulting in a stellate cell configuration characterized by numerous elongated cytoplasmic extensions (Ortiz *et al.*, 1973). Microinjection of cyclic AMP into cultured iris epithelial cells also induces retraction of peripheral cytoplasm coinciding with a reduction in cell to substratum adhesiveness (T. Yamada, 1977). The elevated intracellular level of cyclic AMP in the dorsal iris from days 3 to 8 after lentectomy may, in similar fashion, function in control of the changes in cell shape observed *in vivo* during dedifferentiation: *i.e.* expansion of intercellular spaces through a reduction in cell to cell adhesiveness and the elaboration of microvillar projections associated with depigmentation.

Modulation of enzyme activities:

Alterations in the activities of a number of enzymes within the iris have been demonstrated to occur during the various stages of lens regeneration. Using a histochemical procedure, Ogawa (1962) demonstrated that within the pigmented iris proper the activity of alkaline phosphatase is greatly elevated during dedifferentiation of the pigmented iris epithelial cells and subsequently declines during the period of fiber differentiation within the lens regenerate. The growing lens rudiment itself contained no alkaline phosphatase activity. Using a biochemical assay for

alkaline phosphatase in homogenized iris tissue extracts, Eguchi and Ishikawa (1963) confirmed that there was a rise in enzyme activity within the dorsal iris between the second and tenth days after lentectomy but reported that a relatively brief and less intense elevation in alkaline phosphatase activity occurred within the ventral iris. Ogawa (1962) observed no significant difference in enzyme activity between dorsal and ventral iris sectors by his methods. It has been assumed that the increased alkaline phosphatase activity observed within the iris following lentectomy is related to the enzyme's function in phosphate metabolism during the elevated rRNA synthesis in the early period of lens regeneration (Eguchi and Ishikawa, 1963).

Thorpe *et al.* (1974) have shown that the activity of glutamine synthetase, which catalyzes the conversion of glutamate to glutamine for use in macromolecular assembly, increases six-fold within the iris between the second and fourth days after lentectomy and remains elevated until at least day 6. The timing of the augmentation in enzyme activity suggests that it may bear some relation to the elevated RNA, DNA, and protein synthesis within the iris following lentectomy.

Using a cytochemical test for electron microscopy, T. Yamada *et al.* (1978) have examined the activity of the lysosomal enzyme acid phosphatase during dedifferentiation of iris epithelial cells both *in vivo* and *in vitro*. In the normal iris the enzyme was faintly detectable in only a

small fraction of the iris epithelial cell population. Conversely at six and eleven days after lentectomy, the majority of iris epithelial cells demonstrated acid phosphatase activity in the vicinity of melanosomes as well as within some mitochondria and nuclei. *In vivo*, no obvious difference was apparent between dorsal and ventral iris sectors. The data were interpreted as indicative of increased lysosomal activity during depigmentation and as evidence of autophagy of melanosomes and other intracellular organelles (T. Yamada *et al.*, 1978).

The activity of N-acetylglucosaminidase, an exoglycosidase probably of lysosomal origin, is elevated between days 12 and 27 of lens regeneration (Idoyaga-Vargas and Yamada, 1974). The enzyme activity is higher on the average in the dorsal iris than in the ventral iris and is predominantly associated with the iris epithelium although some activity is present within the iris stroma. In organ culture of dorsal irises considerable N-acetylglucosaminidase activity is released into the culture medium and *in vivo* enzyme activity is present within the ocular fluids during lens regeneration, indicating a potential for the enzyme to function extracellularly (Idoyaga-Vargas and Yamada, 1974). On the basis of the function of N-acetylglucosaminidases in other vertebrate systems, the enzyme could be presumed to be involved in catabolism of the carbohydrate moieties of glycoproteins and proteoglycans. It is not yet established whether the enzyme is elaborated by the iris epithelial

cells themselves or is contained within the large numbers of macrophages which invade the iris during lens regeneration.

The activity of galactosyltransferase, an enzyme which functions in assembly of carbohydrate components of macromolecules, has also been examined (Idoyaga-Vargas *et al.*, 1976). Transferase activity within the dorsal iris proper is significantly elevated at all times during lens regeneration, attaining a maximum between 10 and 17 days after lentectomy. The normal lens exhibits much greater galactosyltransferase activity than the normal iris, while that of the regenerating lens vesicle is higher still. Idoyaga-Vargas *et al.* (1976) suggested that the enzyme functions in assembly of cell-surface glycoproteins, possibly associated with the formation of specialized cell junctions between newly differentiated lens fibers.

Paradoxically, the activity of tyrosinase, an enzyme which catalyzes the conversion of tyrosine into melanin, is enhanced following lentectomy (Achazi and Yamada, 1972). During the first ten days after lentectomy tyrosinase activity increases in the depigmenting dorsal and ventral irises to levels three-fold greater than that of normal iris. Tyrosinase activity increases still further in the tissue of the young lens rudiment. It was suggested that the latter condition might be characteristic of immature lens tissue since high tyrosinase activity was also found in the lenses of young tadpoles during the normal larval

development of *Rana pipiens* (Achazi and Yamada, 1972).

F) *The Initial Stimulus for*

Lens Regeneration:

The foregoing discussion has established that regeneration of the lens in newts proceeds *via* an ordered progression of morphological and biochemical events within the iris epithelium. The question remains as to what specific change in the ocular environment provides the primary stimulus which activates the sequence of regenerative changes within the iris. Early in the study of lens regeneration this problem was addressed in numerous investigations. The results of these studies have been reviewed in considerable detail by Reyer (1954, 1962) and herein will be dealt with only in respect to their most general conclusions.

Wounding:

One possibility, first proposed by Fischel (1900, 1903), is that injury to the iris caused during surgical lens removal might provide the trigger to initiate lens regeneration. However considerable evidence indicates that wounding is neither a sufficient nor a necessary condition for lens regeneration. Lens formation is not stimulated under conditions where the iris is traumatized in the presence of an intact lens (Wolff, 1903; Stone, 1943, 1952), nor when the lens is carefully extirpated and immediately

reimplanted in its normal position within the ocular chamber (Wachs, 1914; Stone, 1943; Zalokar, 1944). Furthermore lens regeneration can be initiated under situations where the lens is not physically removed from the eye at all. Reyer (1948) blocked normal lens formation from head ectoderm in *Triturus* embryos by grafting tissue lacking lens-forming ability in place of the original presumptive lens ectoderm. When the animals attained a stage of larval development wherein iris tissues became fully pigmented, the lens tissue could sometimes regenerate from the dorsal iris margin. Thus neither operative injury nor even the prior presence of a lens was required for initiation of the lens regeneration sequence.

Removal of inhibitory action of lens:

It is undeniable that removal of the original lens of the eye is the event which most regularly succeeds in initiating lens regeneration from the dorsal iris of the newt *in vivo*. A classical concept pertaining to the control of lens regeneration has been that in the normal eye the presence of an intact lens directly inhibits the transformation of dorsal iris epithelium into lens tissue. Removal of the original lens would remove this inhibitory influence and thereby permit the series of regenerative events leading to formation of a new lens. This notion is supported by some experimental evidence. If pieces of dorsal iris are transplanted into the ocular chambers of lentectomized

host eyes they frequently regenerate lens tissue, but fail to do so when grafted into eyes containing intact lenses (Wachs, 1914; Stone, 1943, 1952). The mechanism of the putative inhibitory action of the lens is at present unclear, but data from a variety of studies provide information as to some of its properties:

It is unlikely that simple mechanical pressure exerted by the lens upon the adjacent iris tissue is effective in suppressing lens differentiation within the dorsal iris epithelium. When objects of similar size such as wax spheres (Stone, 1945) or paraffinized lenses (Kesselyak, 1936) are implanted into lentectomized eyes in place of the original lens, no inhibition of lens regeneration is observed. Nevertheless, it appears that contact, or at least the close apposition, of iris and lens tissue is important for exertion of the inhibitory influence of the lens in the normal eye. If the lens is displaced a short distance from the dorsal iris margin by insertion of metal or glass probes (Spirito and Ciaccio, 1931; Eguchi, 1961) or a plio-film membrane (Stone, 1966), lenses may sometimes regenerate from the dorsal iris in the presence of the original lens. Williams (1970) and Williams and Higginbotham (1975) fused the dorsal irises of two newts to produce experimental eyes containing two regeneration-competent zones. After both iris halves had regenerated large lenses, one lens was surgically removed. The remaining lens was capable of inhibiting lens regeneration from the

opposite iridal margin only in those cases where the two tissues lay in close apposition. The role of tissue contacts in suppressing lens regeneration is suggested further by experiments wherein fragments of liver or heart tissue were implanted into lentectomized eyes and were capable of preventing lens regeneration when lying in direct contact with the dorsal iris margin (Mikami, 1941).

The metabolic condition of the lens appears to influence its inhibitory effect on lens regeneration. Alcohol-fixed lenses are unable to inhibit lens regeneration (Wachs, 1914) and cystic or degenerating lenses may retard, but do not prevent, new lens formation (Ikeda and Amatatu, 1941; Uno, 1943; Stone, 1952). In addition, the inhibitory influence of the lens is markedly dependent upon the absolute mass of lens tissue. Reyer (1948) demonstrated that lens regeneration in lentectomized eyes of larval newts could be prevented by embedding large fragments of lens tissue into the retina at the rear of the eyeball, but that fragments of lens tissue beneath a critical size failed to inhibit lens formation. Frost (1961) confirmed that in adult newts the absolute volume of lens tissue, and not its stage of differentiation, determines its competence to suppress lens regeneration in adjacent iris tissue.

A number of experiments suggest that the putative lens inhibition factor may ultimately be attributable to a specific macromolecular constituent of the lens. Stone and Vultee (1949) and Stone (1963) reported that repeated

injections of aqueous humour from eyes containing intact lenses could completely prevent lens regeneration in lenticotomized eyes for periods up to three months. However in similar studies, Goss (1961, 1964) observed no inhibition of regeneration. In other studies, lens tissue extracts have been fractionated by starch gel electrophoresis (Smith, 1965), polyacrylamide gel electrophoresis (Williams and Reynolds, 1977), or agarose gel chromatography (Moore and Williams, 1977). In each case, two or three fractions of the lens extracts retarded lens regeneration when implanted in lenticotomized eyes, while a number of other fractions either failed to or only slightly inhibited lens formation. In keeping with the preceding discussion regarding the role of tissue contacts in inhibition of lens regeneration, it would seem that if a macromolecular lens component acts directly in suppressing lens regeneration from the dorsal iris, it is either not readily diffusible or its concentration rapidly declines below an effective level with increasing distance of the lens from the reacting iris tissue.

Alternatively, the intact lens may inhibit new lens formation in the normal eye through less direct or more subtle mechanisms, e.g. by interfering with the transport to the iris of regeneration stimulatory factors produced by the retina or other ocular tissue. This hypothesis will be discussed in more detail below.

Release of lens promoting factor
from neural retina:

Classical experiments by Spemann at the turn of the century demonstrated that in normal embryonic development of the amphibian eye, the optic vesicle plays a critical role in inducing lens formation from the overlying ectoderm of the embryonic head region (for reviews see Spemann 1938 and Lopashov and Stroeve, 1964). Other studies have confirmed the involvement of the optic cup in embryonic lens formation in birds (Karkinen-Jaaskelainen, 1978) and mammals (Chase and Chase, 1941). Although it is now acknowledged that under certain experimental conditions the exposure of embryonic head ectoderm to the optic cup may be unnecessary, and in other cases insufficient to elicit lens differentiation (McAvoy, 1980), the inductive interaction of the two tissues during normal vertebrate lens development *in situ* is supported by a wealth of experimental evidence. In light of the importance of the optic cup in embryonic lens formation, Spemann suggested that in adult newts its mature derivative, the neural retina, might retain its capacity for induction of lens tissue and stimulate the transformation of iris tissue into lens following lentectomy (see Spemann, 1938). Numerous experiments both *in vivo* and *in vitro* support the hypothesis that a factor released by the neural retina is required for conversion of dorsal iris epithelium into lens tissue. When both the lens and neural retina are simultaneously excised from the

eyes of larval or adult newts the onset of lens regeneration is delayed and subsequently proceeds concurrently with the regeneration of the neural retina from the pigmented retina (Stone and Steinitz, 1953; Powell and Powers, 1973). If isolated pieces of dorsal iris are grafted to extraocular sites such as the body cavity, dorsal fin, or brain ventricle, lens regeneration does not occur unless retinal tissue is included with the explant (Reyer, 1954, 1966; Stone, 1958a). Furthermore lens regeneration in the lenuctomized eye can be blocked by insertion of a physical barrier, such as a cellophane membrane, between the iris and neural retina (Stone, 1958b), by completely filling the vitreous eye chamber with agar, silica gel, or a glass sphere (Zalokar, 1944) or by implanting the chemical adsorbant, kaolin, into the vitreous chamber (Zalokar, 1944). These latter observations suggest that in the normal eye the intact lens might indirectly inhibit lens regeneration by acting as a barrier to diffusion of a retinal stimulating agent, by absorbing the putative retinal factor, or by metabolically neutralizing it (Reyer, 1954).

In organ culture isolated dorsal iris tissue fails to initiate lens vesicle formation in the presence of serum alone, but undergoes depigmentation and lens fiber differentiation in the presence of larval frog neural retina (T. Yamada *et al.*, 1973; T. Yamada and McDevitt, 1974). Alternatively, any of the following can take the place of retinal tissue in promoting lens formation from dorsal iris

in organ culture: the presence of frog or newt pituitaries or spinal ganglia (Connelly *et al.*, 1973), or supplementation of the culture medium with a pituitary thyrotropin preparation (Cuny and Zalík, 1981).

In vivo, lens regeneration from the dorsal iris margin can be elicited even in the presence of an intact lens by implanting retinal tissue into the anterior eyechamber (Williams, 1975) or by similarly inserting whole newt pituitaries (Powell and Segil, 1976). Pieces of dorsal iris grafted into the blastema of the regenerating newt limb form lens-like bodies with high frequency, while the frequency is reduced in denervated limbs (Reyer *et al.*, 1973). The above experiments suggest that lens differentiation from dorsal iris tissue is dependent upon one or more stimulatory factors that under normal circumstances would be supplied by the neural retina but which under experimental conditions can alternatively be supplied by several extraocular tissues. Of interest is the fact that in cultures of dissociated iris epithelial cells, the formation of lens-crystallin synthesizing lentoid bodies is independent of the trophic influence of retinal or pituitary tissues. This implies that integrated iris tissue structure is fundamental to the control of lens regeneration *in vivo*, a matter which will be discussed at length in a succeeding section of this introduction.

It is worthwhile to note that neural retina may also play a role in directing the polarity of the regenerated

lens *in vivo*. When the dorsal iris is rotated 180° to a backward orientation within the lentectomized eye, the new lens will nevertheless regenerate in a normal orientation (lens fibers directed towards the retina and lens epithelium directed towards the cornea) (Stone, 1954). Similarly, if the regenerating lens rudiment itself is re-oriented backwards, its polarity reverses such that lens epithelium now facing the retina elongates to form lens fibers (Reyer, 1974). Probably related is the fact that in lens regeneration in organ culture under the trophic influence of the pituitary gland, the lens fibers of the regenerate are always oriented towards the adjacent pituitary tissue (Connelly *et al.*, 1973) and lens fibers differentiating under the influence of thyrotropin in iris organ culture are always directed towards the periphery of the explant (*i.e.*, towards the surrounding medium containing hormone) (Cuny and Zalik, 1981).

The mode of action of the retina in promoting lens regeneration is at present undetermined. T. Yamada (1977) has described a model under which a retinal factor attenuates the length of the cell cycle of proliferating iris epithelial cells, thereby decreasing the time required for the dorsal iris cell population to complete a critical number of cell divisions required for cell-type conversion. According to Yamada's hypothesis, the retinal factor does not specify the direction of differentiation of the iris epithelial cells. Rather, the tendency for lens fiber

differentiation is considered as an intrinsic property of the pigmented iris cells which can be released under the influence of the retinal factor *in vivo*, by the retina, pituitary, spinal ganglion or thyrotropin in organ culture, or by simply dissociating the pigmented iris epithelial cells and allowing their proliferation in cell culture.

G) *Iris Tissue Organization as a Controlling Element in Lens Regeneration:*

A number of observations suggest that keys to the mechanisms regulating the process of lens regeneration lie in the structural organization of the iris tissue itself. Among the most striking features of lens regeneration *in vivo* is the phenomenon of "dorsal dominance." Under normal conditions lens tissue regenerates only from the dorsal iris, never from ventral iris tissue. Apparently factors intrinsic to the iris tissue determine this distinction. Dorsal iris tissue transplanted to a ventral location retains its competence for lens formation, while ventral iris tissue in the reciprocal transplantation remains unable to regenerate a lens (Stone and Vultee, 1949). Also, when explants of tissue from the different sectors of the iris are grafted into the ocular chambers of lentectomized eyes, pieces of iris from the mid-dorsal region always form lenses at the highest frequency (Sato, 1930; Mikami, 1941). The incidence of lens regeneration declines in explants from dorso-lateral regions, while explants from ventro-

lateral regions form only infrequent depigmented epithelial vesicles. Grafts from the mid-ventral portion of the iris never form lens tissue (Sato, 1930; Mikami, 1941).

Under exceptional experimental conditions, however, cells of the ventral iris epithelium can be induced to undergo cell-type conversion into lens fibers. Ciaccio (1933) reportedly produced supernumerary lenses, some in the ventro-lateral region of the iris, by partitioning the iris with celluloid laminae (see Reyer, 1954). Eguchi and Watanabe (1973) induced multiple lens regenerates, occasionally originating from the ventral iris, by implanting crystals of a potent carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine into the vitreous chamber of lentectomized eyes. Under conditions of organ culture in the presence of either retina or pituitary tissue, explants of complete dorsal iris tissue typically form lenses, while complete ventral iris halves do not (T. Yamada *et al.*, 1973; T. Yamada and McDevitt, 1974; Connelly *et al.*, 1973). However when the fragments of ventral iris epithelium are surgically stripped of their overlying connective tissue stroma, lens tissue is formed in approximately 18% of the ventral epithelium explants (T. Yamada and McDevitt, 1974). Finally, in cultures of dissociated iris epithelial cells, ventral iris epithelial cells undergo cell-type conversion to form crystallin-synthesizing lentoid bodies with a frequency equivalent to that of dissociated dorsal iris cell cultures (Horstman and Zalik, 1974; Eguchi *et al.*, 1974).

A common feature which might underlie each such instance of lens differentiation from ventral iris is a disruption of the normal organization of the iris tissue. In the case of supernumerary lenses produced after partitioning the ventral iris with celluloid septae (Ciaccio, 1933), abnormal tissue reconstruction may result from wound-healing in the presence of an interfering foreign object. Exposure of the ventral iris to a potent carcinogen (experiments of Eguchi and Watanabe, 1973) might in some manner elicit an abnormal disruption of tissue architecture. Removal of the connective tissue stroma of the iris tissue can apparently sometimes release the cells of the ventral epithelium from restrictions normally imposed within the intact tissue with respect to their potential for lens differentiation (T. Yamada and McDevitt, 1974). The procedure of cell dissociation, entailing both destruction of the stromal matrix and disruption of intercellular matrices, adhesive bonds, and cell-surface structure within the iris epithelium, would appear to carry this de-repression a step further. Culture of dissociated epithelial cells provides the only situation where both dorsal and ventral iris cells undergo lens fiber differentiation with equal facility and, as discussed earlier, where this capacity is independent of stimulatory factors required by the intact tissue.

The foregoing suggests that the regulation of phenotypic expression within the iris during lens regeneration

in situ relies upon factors intrinsic to the integrated structure of the iris tissue. The circumstances of lens differentiation from isolated ventral iris epithelium in organ culture and dissociated ventral iris epithelial cells in cell culture indicate that critical elements in this integrated control may reside in the extracellular matrix components and cell-surface materials associated with the iris. It has previously been suggested that the connective tissue stroma of the iris might have a suppressive effect on the transformation of adjacent iris into lens (Lopashov, 1977), perhaps by inhibiting iris epithelial cell proliferation (T. Yamada, 1977). Uhlenhuth (1919) proposed that the rupture of an extracellular membrane associated with the iris might be a critical step in initiating lens regeneration *in vivo* (see Reyer, 1954). Similar ideas have been forwarded more recently in somewhat different forms by Lopashov (1977) and T. Yamada (1977).

In addition to these largely theoretical arguments which support an involvement of cell-surface and extracellular matrix materials in the process of lens regeneration, there is some experimental evidence for such an action. Dedifferentiation of iris epithelial cells both *in vivo* and *in vitro* is associated with a sequential disappearance of cell-surface groups, partially characterized as carbohydrate in nature (Zalik and Scott, 1972, 1973; Zalik *et al.*, 1976). The regenerative events within the iris are further associated with both increased monosaccharide transferase

activity (Idoyaga-Vargas *et al.*, 1976) and increased exoglycosidase activity (Idoyaga-Vargas and Yamada, 1974), indicating that dynamic changes in carbohydrate metabolism may be integral to some of the ultrastructural changes during lens regeneration.

Electron microscopic studies indicate that some of these changes involve surface-associated and extracellular structures, *e.g.*: perforation of the iris epithelial basal lamina, the expansion of intercellular spaces, the elaboration of microvillar cell processes together with shedding of cortical cytoplasm and plasma membrane, and the establishment of specialized lens fiber cell junctions and an acellular lens capsule.

In light of these theoretical and empirical considerations, more detailed analyses of the biochemical changes in the cell-surface and extracellular matrix components of the newt iris during lens regeneration would appear to be warranted. In a wide variety of other developmental systems macromolecules of the cell-surface and pericellular microenvironment have been implicated as regulatory agents in the control of cell division, migration, and gene expression during histogenesis and organ formation (Slavkin and Greulich, 1975; Poste and Nicolson, 1976; Lash and Burger, 1977). Particular attention has been focussed upon a class of extracellular polysaccharides known as the glycosaminoglycans. The biochemistry of these macromolecules as well as some evidence of their roles in the mediation of cellu-

lar interactions during development and growth will be briefly reviewed below.

III. Glycosaminoglycans: Structure, Distribution, and Physiological Roles

A) *Structure:*

The glycosaminoglycans (GAGs), formerly termed acid mucopolysaccharides, comprise a family of predominantly extracellular and cell-surface localized macromolecules that possess certain common structural features. All are polyanionic, linear chains of monosaccharide subunits. Along the length of the GAG polymer, residues of hexosamine (either D-glucosamine or D-galactosamine) typically alternate with residues of uronic acid (D-glucuronic acid or L-iduronic acid). (An exception is keratan sulfate in which D-galactose residues replace the uronic acid moieties characteristic of all other GAGs.) The amino group at the C-2 position of the hexosamine is usually substituted with an acetyl group or, in the case of heparin and heparan sulfate, may instead bear an N-sulfate group. In addition, negatively charged ester sulfate groups may be located at any of several positions on the hexosamine residue or, less frequently, upon the uronic acid moiety. These variously positioned sulfate groups, together with the carboxylic acid groups of the uronic acid residues, confer a high negative charge density to the glycosaminoglycans within the range of physiological pH.

Each of the various types of naturally occurring GAGs is distinguished by a characteristic repeating disaccharide

structural unit. The disaccharide repeat structures of hyaluronate, chondroitins 4- and 6- sulfate, chondroitin, dermatan sulfate, heparin, heparan sulfate, and keratan sulfate are illustrated in Figure 3. These should be regarded as idealized representations since considerable variation from the generalized disaccharide formulae may be encountered in GAGs from different sources. Therefore each of the GAG types might best be considered as a class of closely related macromolecules having some degree of structural heterogeneity.

Hyaluronic acid:

Hyaluronic acid (also known as hyaluronate) is composed of alternating N-acetyl-D-glucosamine and D-glucuronic acid residues (Figure 3a). It is the only widely distributed GAG which bears no sulfate groups on either its hexosamine or uronic acid moieties, and therefore owes its polyanionic properties solely to the carboxylate groups of its glucuronic acid residues. Its primary structure is highly consistent among hyaluronic acids derived from a variety of tissues. The length of the polymer can vary from 100 to 10,000 disaccharide repeat units (*i.e.*, molecular weight of 5×10^4 to 5×10^6 daltons), making hyaluronate the largest of the glycosaminoglycans.

Hyaluronate is a common constituent of connective tissues and is especially enriched in connective tissues with a high fluid content (*e.g.*, umbilical cord, vitreous body

Figure 3.

Structures of the glycosaminoglycans and keratan sulfate, represented by Haworth formulae.

(a) Repeating disaccharide unit of hyaluronic acid: N-acetylglucosamine (GlcNac) joined by a $\beta 1 \rightarrow 4$ glycosidic linkage to glucuronic acid (GlcUA).

(b) Repeating disaccharide unit of chondroitin 4-sulfate: N-acetylgalactosamine 4-sulfate (GalNac 4-SO₄) joined via $\beta 1 \rightarrow 4$ linkage to glucuronic acid (GlcUA).

(c) Repeating disaccharide unit of chondroitin 6-sulfate: N-acetylgalactosamine 6-sulfate (GalNac 6-SO₄) joined via $\beta 1 \rightarrow 4$ linkage to glucuronic acid (GlcUA).

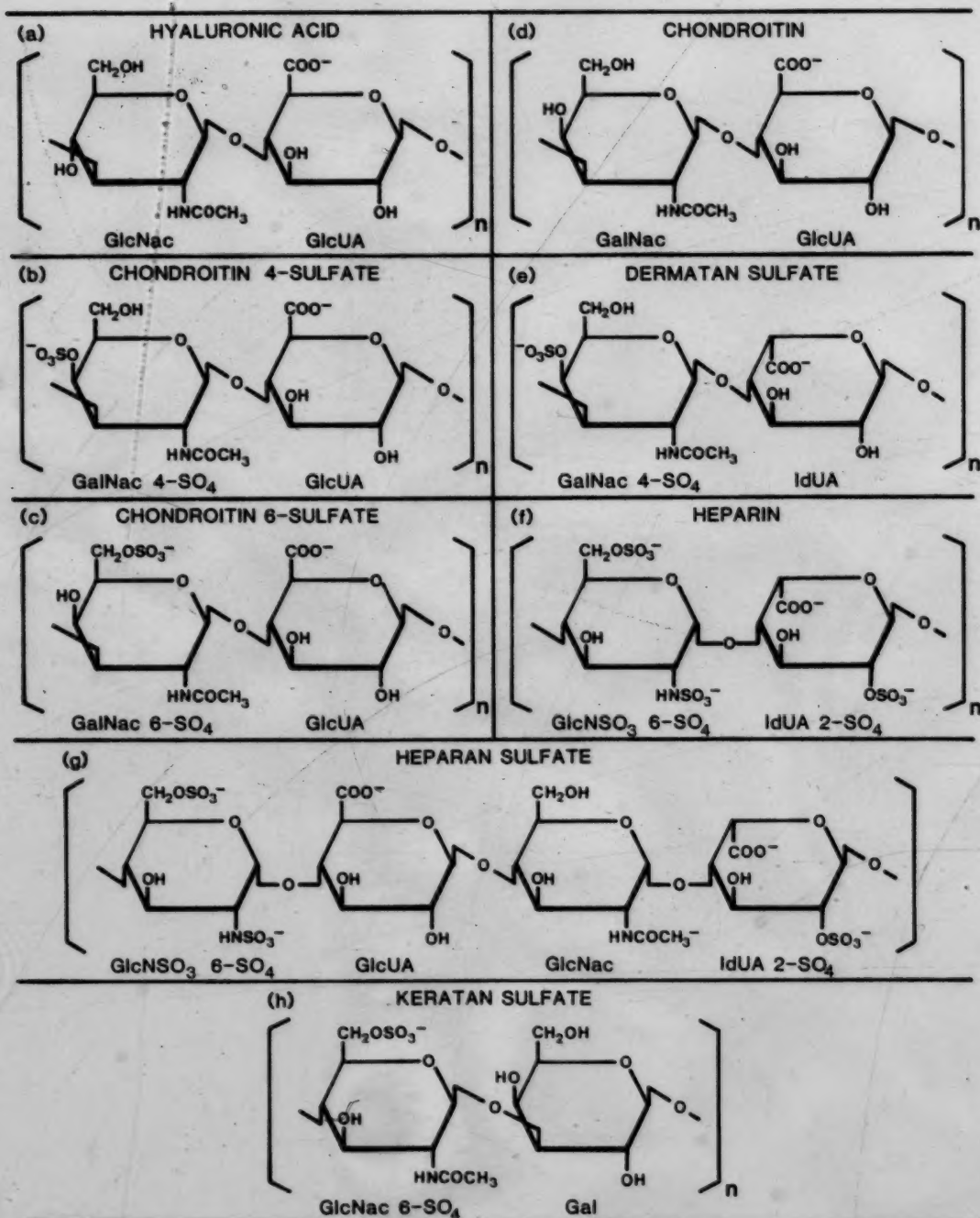
(d) Repeating disaccharide unit of chondroitin: N-acetylgalactosamine (GalNac) joined via $\beta 1 \rightarrow 4$ linkage to glucuronic acid (GlcUA).

(e) Repeating disaccharide unit of dermatan sulfate: N-acetylgalactosamine 4-sulfate (GalNac 4-SO₄) joined via $\beta 1 \rightarrow 4$ linkage to iduronic acid (IdUA). Some iduronate residues may bear an O-sulfate group (not shown).

(f) Repeating disaccharide unit of "idealized" heparin: N-sulfated glucosamine 6-sulfate (GlcNSO₃ 6-SO₄) joined via $\alpha 1 \rightarrow 4$ linkage to iduronate 2-sulfate (IdUA 2-SO₄). Note, however, that natural preparations of heparin demonstrate considerable heterogeneity (see text).

(g) Hypothetical tetrasaccharide region of a heparan sulfate polymer composed of a heterogeneous assemblage of N-sulfated glucosamine 6-sulfate (GlcNSO₃ 6-SO₄), glucuronate (GlcUA), N-acetylglucosamine (GlcNac), and iduronate 2-sulfate (IdUA 2-SO₄).

(h) Repeating disaccharide unit of keratan sulfate: N-acetylglucosamine 6-sulfate (GlcNac 6-SO₄) joined via $\beta 1 \rightarrow 3$ linkage to galactose (Gal). The galactose may bear an O-sulfate group at position C-6 (not shown).



of the eye, synovial fluids of joints, etc.). Its high water-binding potential and visco-elastic properties are presumed to allow hyaluronate to function in maintaining tissue turgor, as an adsorbant against mechanical shock, and as an extracellular lubricant in such matrices (Balazs and Gibbs, 1970; Ogston, 1970; Laurent, 1970). In addition, hyaluronic acid is often abundant in embryonic tissues during periods of morphogenetic movements and cell proliferation (Toole, 1973, 1976), and is sometimes localized at cell surfaces (Underhill and Dorfman, 1978; Mikuni-Takagaki and Toole, 1979) and within embryonic epithelial basement membranes (Cohn *et al.*, 1977; Fisher and Solursh, 1977).

Chondroitin sulfates:

The chondroitin sulfates are assembled from alternating residues of N-acetyl-D-galactosamine and D-glucuronic acid (Figure 3b, c). Chondroitin sulfate polymers are generally shorter than those of hyaluronate, ranging from 20 to 100 repeating disaccharide units in length (molecular weight of 1×10^4 to 5×10^4 daltons). The major GAGs of vertebrate cartilage, chondroitin 4-sulfate (formerly chondroitin sulfate A) and chondroitin 6-sulfate (formerly chondroitin sulfate C), differ only in the location of a single O-sulfate group on the N-acetyl-D-galactosamine residue of the disaccharide repeat unit (at positions C-4 and C-6, respectively). (See Figure 3b, c.) In addition to these

common molecular species, numerous other chondroitin sulfate types with varying degrees of undersulfation, oversulfation, and co-polymeric hybridization have been isolated from vertebrate and invertebrate tissues (Mathews, 1975). Partially sulfated chondroitin sulfates, in which a significant proportion of galactosamine residues lack ester sulfate groups, have been reported (Bettelheim and Philpott, 1960; Kvist and Finnegan, 1970; Juvani *et al.*, 1975; Liao *et al.*, 1978). Indeed, a galactosamine-glucuronate polymer completely devoid of sulfate groups, termed chondroitin, has been identified as a minor component of embryonic corneal and cartilage matrices (Figure 3d) (Davidson and Meyer, 1954; Mathews, 1975). Conversely, there are numerous examples of oversulfated chondroitin sulfates bearing an average of greater than one sulfate residue per disaccharide repeat unit. These involve either sulfation of the glucuronic acid moiety in addition to the usual sulfation on the galactosamine residue (Suzuki, 1960; Mathews, 1975; Seno *et al.*, 1974) or the presence of a second ester sulfate group within the galactosamine moiety (Kawai *et al.*, 1966; Suzuki *et al.*, 1968; Suzuki, 1960). Finally, hybrid polymers containing sequences of chondroitin 6-sulfate disaccharides interspersed within regions of chondroitin 4-sulfate disaccharides are common and in many tissues may be the rule rather than the exception (Seno *et al.*, 1975; Michelacci and Dietrich, 1976; Liao *et al.*, 1978; Faltynek and Silbert, 1978; Antonopoulos *et al.*, 1965).

Chondroitin sulfate GAGs are widely distributed in connective tissue matrices, especially within cartilage, bone, skin, and aortic walls. Also, chondroitin sulfates have been identified in the basement membranes of developing epithelial organs (Cohn *et al.*, 1977) and at cell surfaces of growing tissues and neoplasms (Sampaio *et al.*, 1977). In addition to serving as structural elements in the cartilage ground substance, it has been suggested that the chondroitin sulfates may play roles in directing the growth and deposition of collagen fibrils in intercellular matrices (Kobayashi and Pedrini, 1973; Obrink, 1973b), in regulating the deposition of minerals during endochondral bone formation (Boyd and Neuman, 1951), and in mediating cell recognition and adhesive events during tissue differentiation (Dietrich *et al.*, 1977).

Dermatan Sulfate:

The structure of the disaccharide repeat unit of dermatan sulfate (formerly termed chondroitin sulfate B or β -heparin) differs from that of chondroitin 4-sulfate only in the replacement of D-glucuronic acid with its C-5 epimer, L-iduronate (Figure 3e). As in chondroitin 4-sulfate, the hexosamine is sulfated at position C-4. Dermatan sulfate is the predominant GAG of vertebrate dermis (Meyer and Chaffee, 1941) and is a component of many tissue matrices including blood vessels, heart valves, lung, gastric mucosa, and umbilical cord (Meyer *et al.*, 1956; Smith and

Gallop, 1953; Danishefsky and Bella, 1966). Polymers of dermatan sulfate are similar in average length to those of chondroitin sulfate (molecular weights of 2×10^4 to 5×10^4 daltons) and exhibit marked heterogeneity. All preparations contain at least some D-glucuronic acid, suggesting that probably all dermatan sulfate molecules are co-polymers of dermatan sulfate-type and chondroitin 4-sulfate-type disaccharides (Fransson and Rodén, 1967a, b; Fransson, 1968; Habuchi *et al.*, 1973; Michelacci and Dietrich, 1975). Additional microheterogeneity is introduced by the presence of O-sulfate groups on some iduronate residues in the dermatan sulfates of some tissues (Malmstrom and Fransson, 1971; Fransson *et al.*, 1974). The co-polymeric structure of dermatan sulfate polymers may confer special three-dimensional configuration to the molecules in solution, thereby facilitating their interactions with other matrix macromolecules (Chiarugi *et al.*, 1979).

Heparin and heparan sulfate:

Among the glycosaminoglycans, the most striking degree of structural diversity is found in heparin and the heparan sulfates (Figure 3f, g). While the hexosamine component of the repeating disaccharide is always D-glucosamine, this can be either N-acetylated or N-sulfated (Cifonelli and King, 1972) and can bear additional O-sulfate groups at positions C-6 or C-3 (Lindahl *et al.*, 1977). The uronic acid moiety may be either D-glucuronic acid or L-iduronate,

of which the latter may carry an ester sulfate at the C-2 position (Cifonelli and King, 1977). The glucuronate and iduronate residues are joined to glucosamine *via* special β 1 $\rightarrow$ 4 and α 1 $\rightarrow$ 4 linkages, respectively. These are unique to heparin and heparan sulfate, as all other GAGs contain 1 $\rightarrow$ 3 uronosyl bonds. Heparin and heparan sulfate polymers are typically shorter than other GAGs, being composed of 4 to 40 disaccharide units (*i.e.* molecular weights of 2×10^3 to 2×10^4 daltons).

It is unlikely that any of the naturally occurring heparin and heparan sulfates are homogeneous polymers. Rather the various hexosamine and uronic acid forms tend to be associated in heterogeneous co-polymers. Indeed, there is no clear distinction between heparin and heparan sulfate GAG types, as there are no structural features unique to one molecular class and exclusive of the other. Heparin polymers tend to be characterized by a predominance of sulfated iduronic acid residues over unsulfated glucuronate, by a higher degree of O-sulfation of glucosamine moieties, by a greater level of N-sulfation and a lower degree of N-acetylation. Conversely, the heparan sulfates are comparatively low in overall sulfate content, exhibit more frequent N-acetylation, less N- and O-sulfation, and contain a majority of glucuronate moieties (Roden, 1980).

The unique features of the heparin and heparan sulfate polymers, including the presence of N-sulfate groups and 1 $\rightarrow$ 4 uronosyl bonds, are undoubtedly crucial to their

physiological roles. For example, the anti-coagulant and lipid-clearing pharmacological actions of heparin are not shared by any other GAG types.

While being structurally related, heparin and the heparan sulfates differ in their tissue localizations and, presumably, in biological functions. Heparin appears to be predominantly an intracellular constituent, occurring in the cytoplasmic granules of mast cells from which it may be released during tissue inflammation (Jaques, 1982). By contrast, heparan sulfates are widely distributed components of cell surfaces (Dietrich and DeOca, 1970, 1978; Kraemer, 1971b; Kjellen *et al.*, 1981; Hedman *et al.*, 1982), are found in the basement membranes of renal glomeruli (Kanwar and Farquhar, 1979), sarcomas (Hassell *et al.*, 1980), and epithelial organs (David and Bernfield, 1982), and are the major GAGs of the kidney and liver (Toledo and Dietrich, 1977; Dietrich *et al.*, 1976).

Keratan sulfate:

The structure of keratan sulfate (formerly keratosulfate) is atypical for a glycosaminoglycan in that D-galactose takes the place of a uronic acid moiety in its disaccharide repeating subunit (Figure 3h). As such, keratan sulfate cannot be considered as a true GAG and may be more properly classified as a type of complex oligosaccharide chain of the glycoproteins. The hexosamine moiety of keratan sulfate is N-acetyl-D-glucosamine, generally sulfated

at position C-6. Many of the galactose residues also bear C-6 sulfate groups. However keratan sulfates from different sources, and even within the same tissue, can display marked structural diversity. Molecular weights range from 5×10^3 to 2×10^4 daltons, while total sulfate content is quite variable (Cifonelli *et al.*, 1967; Seno *et al.*, 1965).

Two types of keratan sulfate have been identified. Keratan sulfate I is the predominant polysaccharide of the corneal stroma (Meyer *et al.*, 1953; Hirano *et al.*, 1961) while keratan sulfate II is widely distributed in skeletal tissues such as cartilage and bone (Bray *et al.*, 1967; Mathews and Cifonelli, 1965; Mathews, 1975). The two molecular species differ primarily in the constituents of the linkage regions to protein (Seno *et al.*, 1965; J. R. Baker *et al.*, 1975; Bray *et al.*, 1967; also see below). These include sialic acid, mannose and fucose, monosaccharides commonly present in glycoproteins which further distinguish the keratan sulfates from true glycosaminoglycans (Hirano *et al.*, 1961).

B) Glycosaminoglycans as Components of Proteoglycans:

With the possible exception of hyaluronate, the glycosaminoglycans exist in tissues as covalently associated components of large protein-polysaccharide complexes known as proteoglycans (Roden, 1980). The details of proteoglycan structure have been most thoroughly resolved for the proteoglycans of vertebrate cartilage (Mathews and Lozai-

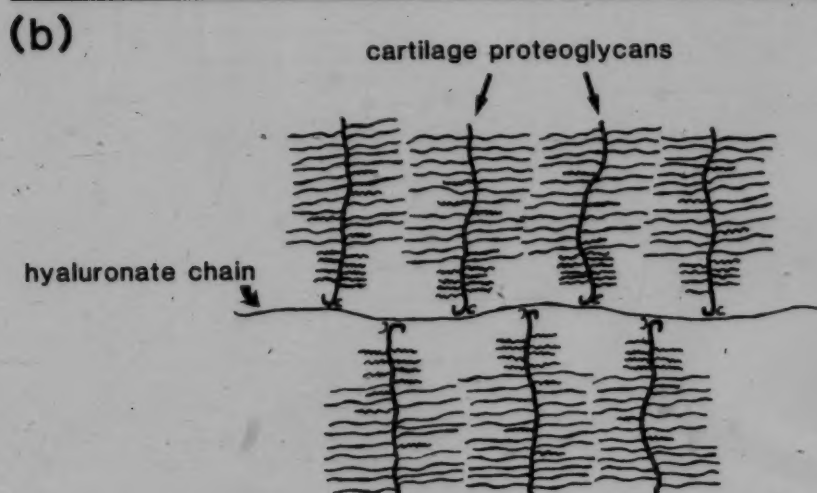
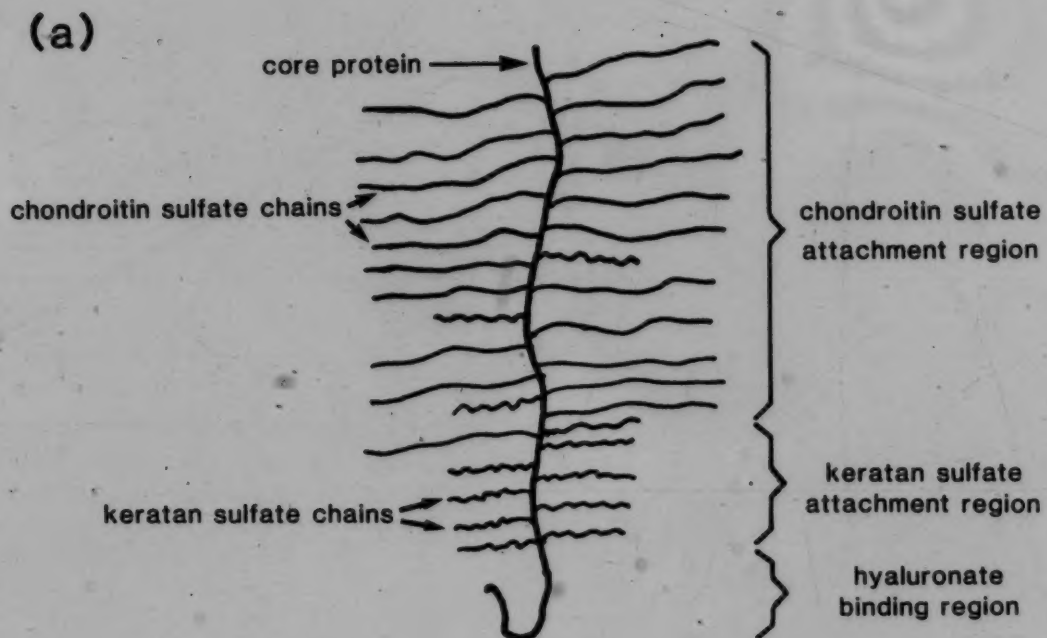
tyte, 1958; Hascall and Heinegard, 1974; Muir and Hardingham, 1975). In these macromolecules approximately 100 chondroitin sulfate chains, with an average molecular weight of 2×10^4 daltons, and about 30 to 60 shorter keratan sulfate II chains ($4-8 \times 10^3$ daltons) are distributed as side-branches along the length of a central core protein, the latter having an average molecular weight of 2×10^5 daltons (Figure 4a). The weight of the entire proteoglycan is approximately 2.5×10^6 daltons, only 8% of which is protein and the remainder carbohydrate. Within the cartilage matrix many such proteoglycan monomers can organize along the length of a hyaluronic acid chain to form a giant proteoglycan aggregate having molecular weight in excess of 2×10^8 daltons (Muir and Hardingham, 1975; Hascall, 1977) (Figure 4b). These proteoglycan aggregates attain sufficient dimensions to be visualized by electron microscopy (Rosenberg *et al.*, 1975).

It is not yet clear to what extent the cartilage proteoglycan can serve as a general model of the structure of proteoglycans in other tissues. Nevertheless, covalently associated protein-GAG complexes of keratan sulfate I (Berman, 1970; Baker *et al.*, 1975), dermatan sulfate (Obrink, 1972), heparin (Horner, 1971; Robinson *et al.*, 1978), and heparan sulfate (Oldberg *et al.*, 1979) have been identified. Hyaluronate generally occurs *in situ* as free polysaccharide chains. Nevertheless, there is evidence that hyaluronate may be covalently linked to protein in synovial

Figure 4.

(a) Model of the proteoglycan monomer of mammalian cartilage. (Adapted from an illustration by Hascall, 1977). About 100 chondroitin sulfate chains and 30-60 keratan sulfate chains are covalently bound to a polypeptide core. The molecular weight of the proteoglycan monomer is approximately 2.5×10^6 daltons.

(b) Model of a cartilage proteoglycan aggregate. (Adapted from an illustration by Hascall, 1977). A number of cartilage proteoglycan monomers are bound to a strand of hyaluronic acid *via* highly specific, non-covalent interactions that involve a hyaluronate-binding region at one end of each proteoglycan core protein. The aggregate may include up to 100 proteoglycan monomers, depending on the length of the central hyaluronate filament.



fluid (Hamerman *et al.*, 1966; Scher and Hamerman, 1972) and that transformed fibroblasts may synthesize hyaluronate on a transient protein core (Mikuni-Takagaki and Toole, 1981). However Prehm (1983a, b) has recently demonstrated that the hyaluronate produced by cultured teratocarcinoma cells is synthesized on intracellular membranes in the absence of any protein primer sequence.

In recent years a concerted effort has been made to elucidate the structures of the linkage regions between the GAG chains and protein cores of proteoglycans (Roden, 1980). The chondroitin sulfates, dermatan sulfate, heparin and heparan sulfate appear to share a common linkage structure, involving attachment of the terminal glucuronic acid residue of the GAG to xylose *via* intermediate galactose residues. The xylose moiety is in turn linked to a hydroxyl group of serine in the core protein (Figure 5a). The keratan sulfates contain structurally different linkage regions, more typical of those of glycoproteins (Figure 5b).

Intracellularly, the production of proteoglycans is envisaged to involve a sequence of biosynthetic events (Roden, 1980):

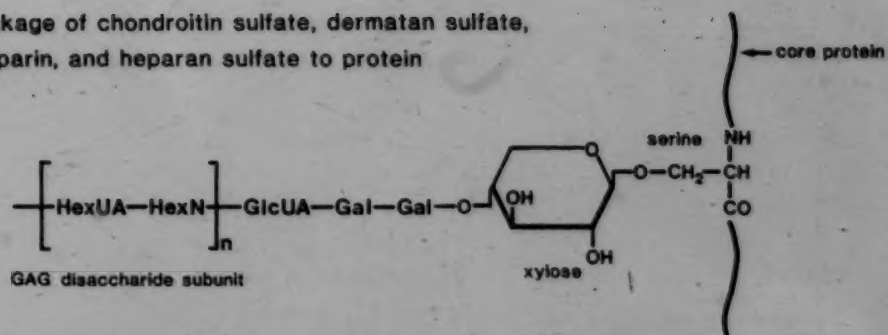
- 1) formation of the protein core on the rough endoplasmic reticulum;
- 2) assembly of the polysaccharide linkage regions through the action of xylosyl and galactosyl transferases (probably within the rough endoplasmic reticulum);
- 3) initiation and elongation of the glycosaminoglycan

Figure 5.

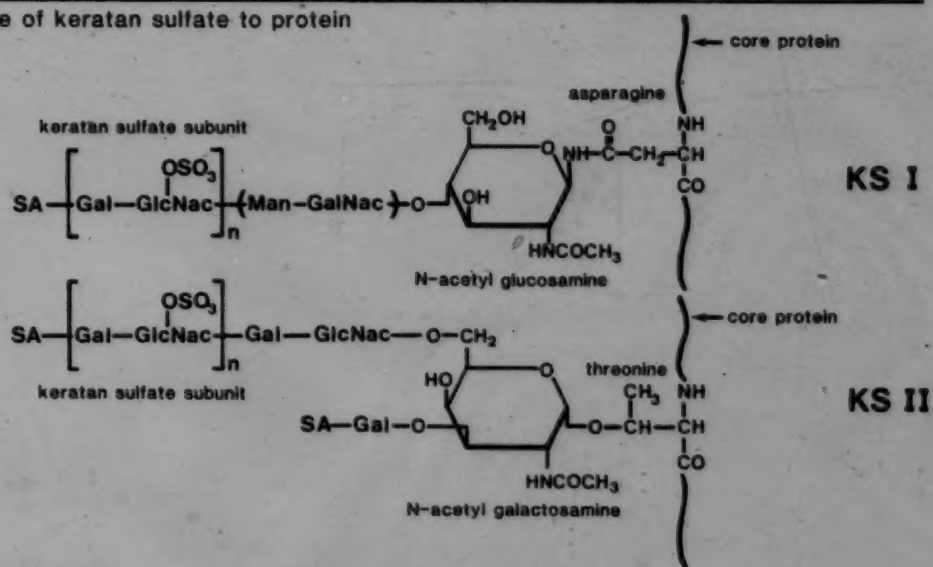
(a) The structure of the linkage regions of chondroitin sulfate, dermatan sulfate, heparin, and heparan sulfate polymers to the core proteins of proteoglycans. The GAG polymer of alternating hexuronic acid (HexUA) and hexosamine (HexN) residues is joined to the hydroxyl group of a serine residue in the polypeptide core *via* an intermediate tetrasaccharide composed of glucuronic acid (GlcUA), two galactose residues (Gal), and xylose. (After descriptions by Roden, 1980).

(b) The possible structures of the linkage regions of keratan sulfate polymers to the core proteins of proteoglycans. (After descriptions by Muir and Hardingham, 1975 and Roden, 1980). In Type I (corneal) keratan sulfate (KS I), the keratan sulfate polymer of alternating galactose (Gal) and N-acetylglucosamine 6-sulfate (GlcNac-OSO₃) residues is joined to the amino group of asparagine in the polypeptide core *via* an intermediate glycan that contains mannose (Man), N-acetylgalactosamine (GalNac) and N-acetylglucosamine residues. The non-reducing terminal of the keratan sulfate polymer bears a sialic acid (SA) residue. In Type II (skeletal) keratan sulfate (KS II), the keratan sulfate polymer is joined to the hydroxyl group of threonine or serine in the polypeptide core *via* intermediate galactose (Gal), N-acetylglucosamine (GlcNac), and N-acetylgalactosamine residues. The linkage region also contains additional galactose and sialic acid residues.

A Linkage of chondroitin sulfate, dermatan sulfate, heparin, and heparan sulfate to protein



B Linkage of keratan sulfate to protein



chains through alternating actions of hexosaminylation and glucuronosyl transferases (in the rough endoplasmic reticulum and Golgi apparatus);

- 4) conversion of D-glucuronate to L-iduronate within the preformed polymer by the action of C-5 epimerase (in dermatan sulfate, heparin and heparan sulfate);
- 5) transfer of O-sulfate and N-sulfate groups to appropriate sites on the polymer by substrate-specific sulfotransferases;
- 6) transfer of the proteoglycan complex to the cell surface or release into the extracellular matrix.

C) *Functions of GAGs in Development and Differentiation:*

The peculiar structural features of the glycosaminoglycans and their parent proteoglycans endow them with specialized physico-chemical properties and the potential for myriad biological functions. In aqueous solution, the GAGs behave as long flexible chains with mutually repulsive electrostatic charges (Ogston, 1970). As such, the polymers occupy much larger spatial domains than do globular molecules of equivalent molecular weight. Within extracellular spaces, meshworks of entwined GAG chains tend to retard the flow of water through tissues, can act as "molecular sieves" in restricting the passage of large macromolecules, and may sterically exclude other molecules from

their extended spatial domains (Laurent, 1966; Ogston, 1970; Comper and Laurent, 1978). The networks of partially immobilized polyanionic chains are further capable of generating strong osmotic potentials which result in local tissue swelling (Ogston, 1970; Muir and Hardingham, 1975). In addition, the high negative charge density of the GAG polymers allows them to interact electrostatically with both small cations and the positively charged domains of macromolecules.

Undeniably, an important biological function of the glycosaminoglycans is to serve as structural elements in maintaining the turgor, water-binding, and elastic properties of connective tissues such as cartilage, dermis, and vitreous humour. Chondroitin sulfate chains act as a major binding site for calcium during endochondral bone ossification (Boyd and Neuman, 1951; Blumenthal *et al.*, 1979), while the electrostatic interaction between GAG and collagen is probably important in stabilizing the fibrillar elements of connective tissue matrices.

Aside from these largely structural functions, however, there is a growing body of evidence which suggests roles for the glycosaminoglycans in mediating cellular motility, adhesive interactions, growth control, and selective gene expression during ontogenetic, regenerative, and neoplastic developmental processes.

During embryogenesis, changes in the amounts and types of GAGs within tissues are often spatially and temporally

correlated with events of cell proliferation, movement, and differentiation. Toole and his co-workers have demonstrated that there is both elevated synthesis and an enriched tissue content of the non-sulfated GAG, hyaluronate, during the periods of mesenchymal cell proliferation and migration associated with morphogenesis in the chick corneal stroma and the cartilaginous structures of the chick limb bud and axial skeleton (Toole and Trelstad, 1971; Toole, 1972). Subsequent differentiation of the mesenchymal cells is preceded by enhanced hyaluronidase activity within the tissues and coincides with an increase in the synthesis of sulfated glycosaminoglycans. A similar pattern of elevated tissue hyaluronate content is observed during the morphogenetic movements associated with neural crest cell migration (Pratt *et al.*, 1975; Derby, 1978; Pintar, 1978), gastrulation and organogenesis in the chick embryo (Solursh, 1976; Solursh *et al.*, 1979; Fisher and Solursh, 1977), formation of the primary mesenchyme and neural folds in the rat embryo (Solursh and Morriss, 1977), heart formation (Markwald *et al.*, 1978; Orkin and Toole, 1978), postnatal brain development (Margolis *et al.*, 1975), and blastema formation in the regenerating adult newt limb (Toole and Gross, 1971).

The sulfated glycosaminoglycans are also dynamic elements during development. Sulfated GAG production is elevated during formation of the primary mesenchyme in the sea urchin embryo (Karp and Solursh, 1974); deposition of

sulfated macromolecules at the ectodermal basal lamina appears to be critical to the migration of these cells during gastrulation (Katow and Solursh, 1981). Histogenesis of the embryonic chick neural retina is associated with an increase in chondroitin sulfate synthesis and a relative decrease in heparan sulfate production, while hyaluronate synthesis remains negligible (Morris *et al.*, 1977). In the embryo of the frog, *Rana pipiens*, enhanced synthesis of sulfated GAGs, primarily heparan sulfate, accompanies cleavage, invagination of the presumptive chordomesoderm at the dorsal lip of the blastopore, and neurulation (Kosher and Searls, 1973). Altered patterns of sulfated GAG production and accumulation have also been demonstrated during corneal development (Meier and Hay, 1973; Hart, 1976), maturation of brain tissue (Margolis *et al.*, 1975), heart morphogenesis (Manasek, 1970; Manasek *et al.*, 1973), cartilage differentiation (Levitt and Dorfman, 1974), and establishment of the secondary palate (Pratt *et al.*, 1973).

Modifications in the production and deposition of GAG also frequently accompany virally induced cell transformation *in vitro* and neoplastic tissue growth *in vivo*. Chiarugi and colleagues have noted reduced amounts of heparan sulfates at the surfaces of some fibroblast cell lines following virus-mediated transformation (Chiarugi *et al.*, 1974, 1981). Alternatively, transformed cells may bear surface-associated heparan sulfates with either a decreased degree of sulfation (Winterbourne and Mora, 1981; Chiarugi

et al., 1981) or altered copolymeric structure (Fransson *et al.*, 1981, 1982). Prior to cell transformation, the surface-associated heparan sulfate may function as a growth regulator in suppressing mitotic proliferation (Chiarugi and Vannucchi, 1976).

The hyaluronate content of the cell surface coat may either increase following transformation (Chiarugi *et al.*, 1981) or decrease (Underhill and Toole, 1982; Mikuni-Takagaki and Toole, 1979) depending on the cell-type and viral transformation agent. A density-dependant increase in chondroitin sulfate production within cultures of normal mouse 3T3-fibroblasts is abolished in Simian Virus-40 transformed lines (Underhill and Keller, 1976), but transformation of liver parenchymal cells induces greater than normal levels of chondroitin sulfate (Ninomiya *et al.*, 1980). Numerous other cases of *in vitro* transformation-dependant changes in GAG have been reported (Cohn *et al.*, 1976; Glimelius *et al.*, 1978a, b; S. R. Baker *et al.*, 1980) as well as qualitatively similar effects in neoplastic tissues *in vivo* (Kojima and Yamagata, 1971; Pillai *et al.*, 1981; Sampaio *et al.*, 1977; Toole *et al.*, 1979; Yamamoto and Terayama, 1973).

A number of experiments *in vitro* suggest possible mechanisms by which GAGs may influence developmental processes. At least in some cell types, surface-associated GAGs may regulate the cell-to-cell and cell-to-substrate adhesions required during morphogenesis and tissue forma-

tion. Heparan sulfates are common constituents of cell surfaces (Kraemer, 1971a, b; Dietrich and DeOca, 1970), where they are apparently associated with proteins in proteoglycan forms (Oldberg *et al.*, 1979). In both hepatocytes and glial cells the heparan sulfate proteoglycans have been shown to exist in two distinct forms: The first type is a proteoglycan which is bound to specific cell-surface receptors and which is readily displaceable with addition of exogenous heparan sulfate. The second type of heparan sulfate proteoglycan is strongly associated with the lipid phase of the plasmalemma and appears to be an integral membrane component (Kjellen *et al.*, 1980, 1981; Norling *et al.*, 1981). These proteoglycan species could interact in several ways to function as ligands in intercellular adhesion (Chiarugi *et al.*, 1979). The heparan-sulfate-binding receptors and the membrane-bound heparan sulfates on apposing cell surfaces might directly form "lock and key" adhesive complexes. Alternatively, two surface receptors on neighbouring cells might bind to a common extracellular heparan sulfate proteoglycan monomer to form a three-component ligand complex.

The adhesion of cells to acellular substrates may also involve heparan sulfates, albeit in a somewhat different manner. Culp and his co-workers have demonstrated that the substrate attachment sites of several cell types cultured *in vitro* are enriched in both heparan sulfate proteoglycans and the adhesion glycoprotein, fibronectin (Rollins and

Culp, 1979; Culp *et al.*, 1979). Specific binding of heparin and heparan sulfate to a domain on the fibronectin molecule has been revealed by affinity chromatography studies (K. M. Yamada *et al.*, 1980; Culp and Dorn, 1982). It is likely that this interaction is important in the fibronectin-mediated adhesion of some cells to acellular substrata both under tissue culture conditions and *in vivo*.

Hyaluronic acid may also modulate the adhesive properties of cells. In some cultured cell lines endogenous cell-surface hyaluronate appears to promote intercellular adhesion (Underhill and Dorfman, 1978; Wright *et al.*, 1981) and substrate attachment (Kraemer and Barnhart, 1978). Lymphoma cells which do not possess endogenous surface-localized hyaluronate may be induced to aggregate by introduction of exogenous hyaluronate to the medium in suspension cultures (Wasteson *et al.*, 1973). The aggregation appears to involve binding of hyaluronate to receptors on apposing cell surfaces. Underhill and Toole (1979, 1980) have demonstrated the presence of specific binding sites for hyaluronic acid on the surfaces of normal and transformed 3T3 fibroblasts. In other cell types, however, endogenous cell-surface hyaluronate may decrease adhesive affinities for natural and artificial substrates (Atherly *et al.*, 1977; Barnhart *et al.*, 1979; Culp *et al.*, 1979; Del Rosso *et al.*, 1981a). Also, in cultures of mouse 3T3-BALB fibroblasts, the addition of exogenous hyaluronate promotes the detachment of cells from their substrates (Abatangelo

et al., 1982).

Even in the absence of cell-surface receptors for GAGs, the polysaccharides may have the capacity to influence the associative interactions of cells. Morris (1979) has demonstrated that cell aggregation *in vitro* can be promoted by the steric exclusion of cells from the domains occupied by meshworks of GAG polymers.

Another manner in which glycosaminoglycans may be capable of influencing developmental processes is through modifying the synthetic activities of cells. It has been repeatedly demonstrated that the presence of exogenous hyaluronic acid in cultures of chondroblasts or mature chondrocytes inhibits both the synthesis of cartilage-specific proteoglycans (Toole, 1973; Wiebkin and Muir, 1973; Solursh *et al.*, 1974; Handley and Lowther, 1976) and deposition of these proteoglycans into the pericellular matrix (Solursh *et al.*, 1980). Conversely, supplementation of cultures with exogenous cartilage-type proteoglycans stimulates the synthesis of cartilage proteoglycans by chondrogenic cells *in vitro* (Nevo and Dorfman, 1972; Kosher *et al.*, 1973; Kosher and Lash, 1975; Handley and Lowther, 1977; Lash and Vasan, 1978). In similar fashion, the addition of exogenous chondroitin sulfate and heparan sulfate to the culture medium of isolated chick embryonic corneal epithelium reinforces its production of chondroitin and heparan sulfate GAGs (Meier and Hay, 1974). This type of "positive-feedback" may be important in coordinately stimu-

lating the GAG synthetic activities of neighbouring cells during the formation of extracellular matrix structures.

Glycosaminoglycans are now recognized as components of the basement membranes underlying many embryonic and adult epithelia. Elegant studies by Bernfield and his colleagues have established that the integrity of the GAG constituents of the epithelial basement membrane is critical to the development and stabilization of branching morphology in organ cultures of embryonic mouse salivary glands (Bernfield *et al.*, 1972; Cohn *et al.*, 1977). In the specialized basement membrane of the adult renal glomerulus, heparan sulfate proteoglycans play an important role in restricting the permeability of the glomerular complex to anionic macromolecules (Kanwar *et al.*, 1981; Farquhar, 1981). It has been suggested that the basement membranes of embryonic epithelia may similarly function in regulating the passage of morphogens through tissues, and may thereby mediate inductive tissue interactions during development (Hay, 1978).

Recent investigations have revealed the capacity for GAGs to interact *in vitro* with a wide range of biological macromolecules (Lindahl and Hook, 1978; Chakrabarti and Park, 1980). A variety of analytical methods have demonstrated the electrostatic binding of sulfated glycosaminoglycans (excepting keratan sulfate) and their parent proteoglycans to collagen, the major fibrillar element of extracellular matrices (Mathews, 1965; Toole and Lowther, 1968a, b; Obrink, 1973a, 1975; Obrink *et al.*, 1975; Gelman

and Blackwell, 1974). In addition, the presence of GAG or proteoglycan can influence the rate of collagen fibril formation *in vitro* (Lowther and Natarajan, 1972; Obrink, 1973b; Oegema *et al.*, 1975). In connective tissues, a correlation has been established between the type of collagen within matrices and the predominant molecular species of interstitial glycosaminoglycan (Junquiera *et al.*, 1981).

Fibronectin, a glycoprotein present in soluble form in plasma and in insoluble form at the surfaces of many cell types, has been shown to possess binding sites for heparin (Stathakis and Mosesson, 1977; Perkins *et al.*, 1979; Masao *et al.*, 1980; K. M. Yamada *et al.*, 1980), hyaluronic acid (K. M. Yamada *et al.*, 1980), and heparan sulfate (Culp and Domen, 1982). The presence of heparin appears to strengthen the interaction between fibronectin and collagen (Ruoslahti and Engvall, 1980; Johansson and Hook, 1980).

Laminin, a major glycoprotein of many epithelial basement membranes, has a strong binding affinity for the copolymeric GAGs, dermatan sulfate, heparin, and heparan sulfate (Del Rosso *et al.*, 1981b).

Interactions of heparin and heparan sulfates with the plasma proteins thrombin, antithrombin, coagulation factors, and serum lipoproteins are well established and may be important in hemostasis (Lindahl and Hook, 1978).

The binding of GAG chains to the core proteins of

proteoglycans has already been discussed, as has the capacity of cartilage proteoglycans to aggregate with hyaluronic acid to form supramolecular complexes (see section III-B of this Introduction).

A peculiar feature of some GAG chains is the capacity for identical polymers to bind directly to one another. This capacity for "self-association" appears to depend upon the special conformational characteristics of copolymeric GAGs that contain both glucuronate and iduronate residues (e.g., dermatan sulfate, heparan sulfate, and heparin) (Coster *et al.*, 1981; Fransson *et al.*, 1981). Chain interactions between hyaluronate and chondroitin sulfates have also been described (Turley and Roth, 1980).

Finally it should be mentioned that there are reports of GAG localization within cell nuclei (Bhavanandan and Davidson, 1975; Stein *et al.*, 1975; Fromme *et al.*, 1976). Experimental evidence suggests that the GAGs, particularly heparin types, can induce changes in chromatin structure (Saiga and Kinoshita, 1976; Hildebrand *et al.*, 1977). Thus, the potential exists for direct effects of intracellular GAG upon genomic expression.

It is likely that the physiological roles of the glycosaminoglycans and proteoglycans *in vivo* are intimately related to their extensive abilities to interact with a wide range of macromolecules which perform regulatory functions within tissues.

METHODS

I. Materials and Sources

A) *Animals:*

Adult newts of the species *Notophthalmus viridescens* were purchased from Lee's Newt Farm, Oak Ridge, Tennessee. The specimens had been collected from naturally breeding populations (William Lee, personal communication). In the laboratory, the animals were fed weekly and maintained at 5-10°C in aquaria containing dechlorinated water until needed for experimental purposes.

To obtain specimens at a desired stage of lens regeneration, each newt was anaesthetized in 0.05% Tricaine (ethyl m-aminobenzoate methanesulfonic acid) and then lentectomized by making a horizontal incision across the cornea of the eye and gently squeezing the eyeball to express the lens. Following surgery, animals were reared at room temperature (20-22°C) for the time interval required to attain the desired stage of lens regeneration (e.g. for 5, 10, 15, 20, or 30 days after lentectomy).

As controls representing the condition of the untraumatized eye, groups of normal unoperated newts were used. In other cases (where specified below) the controls involved "sham-lentectomy" of the eyes by making a horizontal incision across the cornea while leaving the lens *in situ*.

B) *Chemicals, Enzymes, Isotopes, and Solutions:*

- N-acetyl-D-galactosamine [Sigma Chemical Co.]
- N-acetyl-D-glucosamine [Sigma Chemical Co.]
- Amphibian Ringer: prepared by dissolving 6.5 g NaCl, 0.14 g KCl, 0.12 g CaCl₂, 0.09 g NaH₂PO₄·H₂O, 0.62 g Na₂HPO₄, 1.8 g glucose, and 0.1 g MgSO₄·7H₂O in distilled water to a final volume of 1 liter.
The solution was adjusted to pH 7.55, if necessary.
- Aquasol liquid scintillation cocktail [New England Nuclear Canada Ltd.]
- Bio-Rad protein assay dye reagent concentrate [Bio-Rad Laboratories]
- 10% buffered formalin containing 0.5% CPC: prepared by dissolving 100 ml of 37% aqueous formaldehyde, 4 g NaH₂PO₄·H₂O, 6.5 g Na₂HPO₄, and 5 g cetylpyridinium chloride in distilled water to a final volume of 1 liter.
- Carbowax-PEG 20,000 (polyethylene glycol) [Fisher Scientific Co.]
- cetylpyridinium chloride (CPC) [Sigma Chemical Co.]
- chondroitinase ABC (from *Proteus vulgaris*) [prepared by Seikagaku Kogyo Co. Ltd.; obtained from Miles Laboratories Inc.]
- chondroitinase AC (chondroitinase AC-II; from *Arthrobacter aurescens*) [prepared by Seikagaku Kogyo Co. Ltd.; obtained from Miles Laboratories Inc.]
- chondroitin 4-sulfate (chondroitin sulfate A, sodium

- salt; from whale cartilage) [prepared by Seikagaku Kogyo Co. Ltd; obtained from Miles Laboratories Inc.]
- chondroitin 6-sulfate (chondroitin sulfate C, sodium salt; from shark cartilage) [prepared by Seikagaku Kogyo Co. Ltd.; obtained from Miles Laboratories Inc.]
 - dialysis tubing (molecular weight retention of approximately 1000 daltons) (Spectra/Por 6-wet tubing; MWCO 1,000) [Spectrum Medical Industries Inc.]
 - 1:5 diluted Tris buffer: prepared by diluting one volume of enriched Tris buffer with four volumes of distilled water.
 - p-dimethylaminobenzaldehyde [J. T. Baker Chemical Co.]
 - enriched Tris buffer: prepared by dissolving 3.0 g Tris (hydroxymethylaminomethane), 2.4 g sodium acetate, 1.46 g NaCl, and 0.05 g BSA in distilled water to a final volume of 100 ml. Adjusted to desired pH with HCl. (According to method of Saito *et al.*, 1968).
 - gentamicin (gentamicin sulfate; 10 mg/ml aqueous solution) [Sigma Chemical Co.]
 - D-[6-³H(N)]-glucosamine hydrochloride (specific activity of 19.0-20.2 Ci/mmol; in sterile aqueous solution) [New England Nuclear Canada Ltd.]
 - heparan sulfate [prepared by Upjohn Pharmaceuticals; obtained as a generous gift from G. Gibson, Dept.

of Oral Biology, University of Alberta]

- heparin (sodium salt, Grade I; from hog intestinal mucosa) [Sigma Chemical Co.]
- hyaluronic acid (from human umbilical cord) [Miles Laboratories Inc.]
- Kodak *Dektol* [Eastman Kodak Co.]
- Kodak *NTB-3* nuclear track emulsion [Eastman Kodak Co.]
- Kodak *Rapid Fixer* [Eastman Kodak Co.]
- NCS tissue solubilizer [Amersham Corp.]
- papain (crude) [Calbiochem Corp.]
- papain (2x crystallized, Type IV) [Sigma Chemical Co.]
- *Phasar* liquid scintillation cocktail [Amersham Corp.]
- POPOP (p-Bis[2-(5-phenyloxazolyl)]benzene) [New England Nuclear Canada Ltd.]
- PPO (2,5-diphenyloxazole) [New England Nuclear Canada Ltd.]
- $^{35}\text{S}[\text{H}_2\text{SO}_4]$ (carrier-free; in aqueous solution) [New England Nuclear Canada Ltd.]
- Sorvall Methacrylate Embedding Medium [Dupont Instruments]
- *Streptomyces* hyaluronidase (hyaluronidase, fungal, from *Streptomyces hyalurolyticus*) [Calbiochem-Behring Corp.]
- Tricaine (ethyl m-aminobenzoate methanesulfonic acid) [Sigma Chemical Co.]
- trichloroacetic acid (TCA) [Sigma Chemical Co.]

II. Biochemical Analyses of $^{35}\text{SO}_4$ Incorporation into Sulfated GAGs of Iris and Lens Tissues

A) Rationale:

With the exceptions of hyaluronate and unsulfated chondroitin, all GAGs bear one or more sulfate groups on their hexosamine or iduronic acid moieties (see INTRODUCTION, section III-A). During GAG biosynthesis, these sulfate groups are derived from the intracellular pool of inorganic sulfate, are incorporated into the metabolic intermediate 3'-phosphoadenosyl 5'-phosphosulfate (PAPS), and are transferred to their final positions on freshly polymerized regions of elongating GAG chains (Sharon, 1975). In vertebrates, the range of possible metabolic fates for molecules of inorganic sulfate is fairly limited. The majority of inorganic sulfate administered to experimental animals is rapidly excreted while most of the remaining sulfate is incorporated into the carbohydrate moieties of glycosaminoglycans and of some glycoproteins and glycolipids (Dziewiatkowski, 1958; De Meio, 1967). Inorganic sulfate is not incorporated into vertebrate protein since the sulfur groups of proteins are derived from the essential amino acid methionine and its derivative cysteine rather than from the intracellular sulfate pool (Huovinen and Gustafsson, 1967). Thus, owing to its very specific pathways of incorporation in the synthesis of biological macromolecules, inorganic sulfate bearing the radioactive

isotope ^{35}S (i.e., $^{35}\text{SO}_4$) serves as a convenient labelled precursor for use in studies of sulfated GAG production in vertebrate tissues.

The following procedures were used to measure the relative levels of incorporation of $^{35}\text{SO}_4$ into the sulfated GAGs of newt iris and lens tissues at various stages of lens regeneration. The types of sulfated GAGs in which the label was bound were determined using a series of chemical and enzymatic hydrolytic treatments having different specificities for GAG substrates.

B) *Administration of $^{35}\text{SO}_4$ to Experimental Animals:*

In each experiment 25-30 newts were bilaterally lentectomized as described above. The animals were maintained for a period of 5, 10, 15, 20, or 30 days after lentectomy to attain the desired stage of lens regeneration. At 48 hours immediately prior to the end of this post-operative rearing period, each newt was administered an intraperitoneal injection of 10 μCi of carrier-free $[^{35}\text{S}]\text{H}_2\text{SO}_4$. (In preliminary experiments, labelling periods of 24, 48, and 72 hours were compared on groups of 10-15 animals.) Following the period of precursor uptake (48 h unless specified otherwise), the newts were anaesthetized, their iris and lens tissues were excised, and the animals were killed.

As controls representing the normal condition of the untraumatized eye, groups of 25-30 unoperated animals were administered $[^{35}\text{S}]\text{H}_2\text{SO}_4$ in identical fashion. Following an

equivalent labelling period, the normal iris and lens tissues were excised and the animals were killed.

C) *Isolation of Iris and Lens Tissues:*

The iris together with the cornea was dissected from each eye of each anaesthetized newt by cutting around the circumference of the eye just posterior to the junction of the cornea and sclera. The irido-corneal complexes were transferred to ice-cold Amphibian Ringer (pH 7.55) where each iris was stripped from the cornea and cleaned of any adherent retinal tissue. When dealing with lentectomized animals at the various regeneration stages, any lens tissue present at the dorsal iris margin was included with the excised iris tissue. In groups of control animals the normal iris and lens tissues were collected separately. The isolated tissues were rinsed in fresh Ringer saline and transferred to cold acetone. The 50-60 irises from animals of each experimental group were pooled for subsequent analysis.

In the majority of experiments whole iris rings were harvested. In experiments designed to examine regional differences in isotope incorporation, the irises were divided into dorsal halves (containing any newly regenerated lens tissue) and ventral halves. The lenses from groups of normal control animals and from one group of 30-day regenerate newts were collected independently as well.

D) *Preparation of Iris/Lens Tissue Homogenates:*

The pooled tissue fragments from each group of animals were transferred to acetone, shaken, and allowed to stand at room temperature for at least two hours to extract lipids. The acetone was withdrawn, the tissue was dried overnight under a vacuum, and its lipid-free dry weight was measured. This was typically 4 to 6 mg for 60 pooled whole iris rings and 16 to 20 mg for the pooled lenses of normal control animals. The acetone-dried tissue was transferred to a glass homogenizing tube and homogenized in 1 ml of 0.1 M sodium phosphate buffer (pH 6.5) using a motor-driven teflon or ground-glass pestle. The homogenate was immediately boiled for 20-30 min to deactivate endogenous enzymes and to denature the tissue collagens. Upon cooling the homogenate was supplemented with 1 ml of a solution of 0.1 M sodium phosphate/0.01 M EDTA/0.02 M cysteine (pH 6.5).

E) *Preparation of Ethanol-Insoluble GAG-Enriched Fraction from Iris/Lens Tissue Homogenates:*

In order to degrade proteins in the tissue homogenate, the papain digestion procedure of Scott (1960) as modified by Toole and Gross (1971) was followed. Papain (2×crystallized) was dissolved in a solution containing 0.1 M sodium phosphate/0.005 M EDTA/0.01 M cysteine at a concentration of 1 mg/ml. The papain solution was activated by incubating it at 65°C for 15-30 min. To the tissue homogenate was added 0.5 ml of the pre-activated papain solu-

tion and the mixture was incubated 24 h at 65°C. After addition of 0.5 ml of fresh pre-activated papain solution, the proteolysis was continued for a further 24 h. The final digest was boiled 5 min and cleared by centrifugation (900 g; 30 min). The supernatant was collected; the pellet was subjected to alkaline hydrolysis in 1 ml of 0.5 N NaOH for 24 h at 4°C on a rotatory shaker to degrade residual protein and ensure maximal liberation of bound polysaccharides. The alkali-soluble digest was cleared by centrifugation (900 g; 30 min) and combined with the supernatant of the prior papain digestion. Trichloroacetic acid (TCA) was added to a final concentration of 10%. The precipitates which formed overnight at 0°C were removed by centrifugation (10,000 g; 30 min). The TCA-soluble supernatant was neutralized with NaOH and supplemented with carrier GAG (a mixture of 100-250 µg each of chondroitin 4-sulfate, chondroitin 6-sulfate, heparin, and heparan sulfate). The carrier and native GAGs were precipitated from solution by the addition of four volumes of 5% potassium acetate in 95% ethanol, following the method of Roden *et al.* (1972). The polysaccharide precipitates that formed overnight at 5°C were collected by centrifugation (10,000 g; 45 min) and redissolved in 2 ml distilled water. The sequence of precipitation with ethanolic potassium acetate and redissolution in water was repeated four times to yield a crude ethanol-insoluble GAG-enriched fraction. This fraction was processed further according to one of the two following

protocols:

F) *Measurement of Total $^{35}\text{SO}_4$*

Incorporation into GAG:

The following procedure was used to measure the total amount of $^{35}\text{SO}_4$ label incorporated into the GAGs of iris and lens tissues at the various regeneration stages. A crude ethanol-insoluble GAG-enriched fraction was prepared from each iris/lens tissue homogenate in the presence of 800-1000 μg carrier GAG, by the methods described above. This fraction was dissolved in 4 ml distilled water, after which 1 ml of 0.5% aqueous cetylpyridinium chloride (CPC) solution and 5 ml of 0.1% CPC in 0.04 M sodium sulfate were added. Overnight incubation at 37°C promoted the formation of flocculent precipitates of CPC-GAG complexes, as described by Scott (1960). The precipitates were collected by centrifugation (10,000 g; 45 min; 25-30°C), washed by re-suspending in 10 ml of 0.05% CPC in 0.02 M sodium sulfate, and recovered by a second centrifugation. The pellet was dissolved in 1 ml NCS tissue solubilizer by incubating 3 h at 50°C with occasional shaking. The sample was cooled, neutralized with 34 μl acetic acid, and added to 14 ml of scintillation cocktail (4 g PPO/0.125 g POPOP per litre of toluene). As an alternate procedure, in some experiments the pellet was dissolved in 0.5 ml 5 N HCl and added to 15 ml of Phasar scintillation cocktail. Measurements of total ^{35}S radioactivity in each sample were made

on either a Nuclear Chicago Mark II or Beckman LS-9000 model liquid scintillation counter. Sample quenching was monitored by sample channels ratio or H-number methods. Counting efficiencies were determined by addition of [^{35}S]H₂SO₄ internal standards or from quench correction curves. Efficiency was typically 70-90% under the counting conditions described.

G) *Identification of Relative Amounts of $^{35}\text{SO}_4$
Incorporated by Different Types of GAG:*

The various classes of sulfated GAG differ in their susceptibilities to degradation by nitrous acid and the enzymes chondroitinase AC and chondroitinase ABC (Taniguchi, 1976). Chondroitinase AC, an endoglycosidase of bacterial origin, is capable of degrading both chondroitin 4-sulfate and chondroitin 6-sulfate to their sulfated disaccharide subunits (Yamagata *et al.*, 1968). The non-sulfated GAG hyaluronate is also degraded, albeit at a reduced rate. Dermatan sulfate molecules bearing only iduronate-type uronic acid moieties are not degraded by chondroitinase AC, but hybrid dermatan sulfate polymers are cleaved at the sites of glucuronate residues (Fransson, 1968). The enzyme chondroitinase ABC degrades iduronate-rich sections of dermatan sulfate as well as polymers of chondroitins 4- and 6-sulfate (Yamagata *et al.*, 1968). Nitrous acid selectively cleaves glycosidic linkages of hexosamines bearing N-sulfate groups (Lagunoff and Warren, 1962). The latter are

peculiar to GAGs of the heparin and heparan sulfate types, which are degraded by the acid treatment. Conversely, the chondroitins 4- and 6-sulfate, dermatan sulfate, and hyaluronate remain intact.

The differing specificities of these hydrolytic treatments were employed to identify the relative amounts of $^{35}\text{SO}_4$ label incorporated by three broad classes of GAG: the chondroitins 4- and 6-sulfate (C4S/C6S), dermatan sulfate (DS), and heparin/heparan sulfate (Hep/HS). A crude ethanol-insoluble GAG-enriched fraction was prepared from each iris/lens tissue homogenate as described above (METHODS--section II-E), with the exception that only 400 μg carrier GAG was used. The ethanol-insoluble fraction was dissolved in 2 ml distilled water and divided into five aliquots for treatment as outlined in Figure 6. Each aliquot (400 μl) was supplemented with 100 μl of enriched Tris buffer (METHODS--section I-B) and incubated with either chondroitinase AC-II (0.5 unit in 100 μl of 1:5 diluted Tris buffer), chondroitinase ABC (0.5 unit in 100 μl of 1:5 diluted Tris buffer), or 100 μl of 1:5 diluted Tris buffer containing no enzyme (mock enzyme digestion). The buffers were used at pH 7.3 (for chondroitinase AC treatment) or pH 8.0 (for chondroitinase ABC and mock digestions). All incubations were for 3 h at 37°C, after which samples were boiled for 5 min.

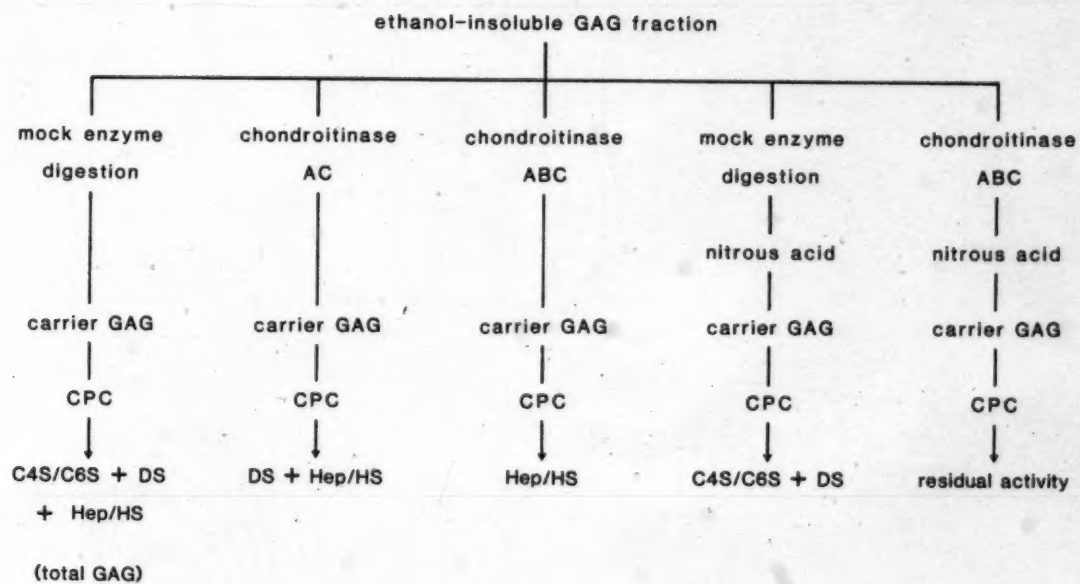
Two aliquots were subjected to subsequent hydrolysis with nitrous acid, following the method of Lagunoff and

Figure 6.

Scheme for identifying the relative amounts of $^{35}\text{SO}_4$ incorporated into three classes of sulfated GAG. The ethanol-insoluble GAG fraction from each iris homogenate was divided into equal aliquots for treatment with chondroitinase AC, chondroitinase ABC, nitrous acid, or both chondroitinase ABC and nitrous acid. A control aliquot underwent mock digestion in enzyme buffer alone. In the presence of carriers, all non-degraded GAG was precipitated with CPC for measurement of bound ^{35}S label. The types of sulfated GAG which are recovered after each treatment are indicated in the bottom row.

Abbreviations:

C4S/C6S	= chondroitins 4- and 6-sulfate
DS	= dermatan sulfate
Hep/HS	= heparin and/or heparan sulfate
residual	= CPC-precipitable label not attributable to GAGs of the C4S/C6S, DS, or Hep/HS types



Warren (1962): samples were incubated 90 min at room temperature in the presence of equal volumes (600 μ l each) of 5% sodium nitrite and 33% acetic acid. Following nitrous acid hydrolysis, samples were neutralized with NaOH.

After each treatment non-degraded GAG was precipitated by the addition of 12 ml of 0.1% CPC in 0.02 M sodium sulfate. An additional 400 μ g of carrier GAG was added to each sample. After overnight incubation at 37°C, CPC-GAG precipitates were collected by centrifugation, washed, and prepared for liquid scintillation counting as described above (METHODS--section II-F).

The different types of GAG and their relative levels of $^{35}\text{SO}_4$ incorporation were identified according to the following scheme (see Figure 6): Radioactivity in total sulfated GAG was determined from counts remaining after mock digestion alone. The proportion of label incorporated into chondroitins 4- and 6-sulfate (C4S/C6S) was estimated from the difference between counts in total GAG and counts remaining after chondroitinase AC treatment. Incorporation into dermatan sulfate (DS) was estimated from the difference between chondroitinase AC- and ABC- resistant counts. Label in heparin and/or heparan sulfate (Hep/HS) was calculated by two methods: from the counts remaining after chondroitinase ABC digestion and from the difference between counts in total GAG and counts remaining after nitrous acid hydrolysis. The values derived by these two methods agreed to within $8 \pm 3\%$ (mean $\pm 95\%$ confidence limits). The arith-

metic mean of the two values was used as a measure of Hep/HS incorporation. The residual radioactivity after a sequential treatment with both chondroitinase ABC and nitrous acid provided an estimate of the proportion of label not attributable to GAG of the C4S/C6S, DS, and Hep/HS types.

III. Biochemical Analyses of ^3H -Glucosamine Incorporation
into GAGs of Newt Iris/Lens Tissue:
Labelling of Hyaluronate

A) *Rationale:*

The utility of $^{35}\text{SO}_4$ as a radioactive precursor in studies of GAG biosynthesis is limited to the extent that non-sulfated GAGs, specifically hyaluronate and non-sulfated chondroitin, do not incorporate this label. To allow quantitative estimates of the levels of hyaluronate production within the newt iris during lens regeneration, an experimental protocol was followed which employed ^3H -glucosamine, a monosaccharide GAG-precursor bearing the radioactive hydrogen isotope tritium (^3H).

According to recognized pathways of carbohydrate metabolism in vertebrate tissues, labelled glucosamine can be assembled into the repeating disaccharide backbone structures of all known types of GAG. The glucosamine is incorporated either in the form of its acetylated derivative N-acetylglucosamine (in hyaluronate, heparin, heparan sulfate, and keratan sulfate) or in the form of N-acetylgalactosamine (in chondroitin, chondroitins 4- and 6-sulfate, and dermatan sulfate) following an intracellular C-4 epimerization reaction (Roden and Schwartz, 1975).

The proportions of ^3H -glucosamine label incorporated into hyaluronic acid in newt iris tissues were determined by utilizing the capacity of a fungal endoglycosidase,

Streptomyces hyaluronidase, to selectively degrade hyaluronate while leaving GAGs of all other types intact (Ohya and Kaneko, 1970).

B) *Administration of ^3H -Glucosamine to Experimental*

Animals and Isolation of Iris/Lens Tissues:

For each experiment, 50-60 newts were used. The right eyes of the animals were lentectomized as described previously (METHODS--section I-A). In order to serve as controls, the left eyes of the same animals were sham-lentectomized by making a horizontal incision across the cornea while leaving the lens *in situ*. The animals were maintained at room temperature for 5, 10, 15, 20, or 30 days following surgery in order to attain the desired stage of lens regeneration. At 24 hours prior to the end of the post-operative rearing period, each newt was given an intraperitoneal injection of 10 μCi of D-[6- $^3\text{H}(\text{N})$]-glucosamine hydrochloride (specific activity of 19.0-20.2 Ci/mmol). Following the 24-hour labelling period, the newts were anaesthetized in 0.05% Tricaine. Iris and lens tissues were excised as previously described (METHODS--section II-C). Dorsal iris halves (including any newly regenerated lens tissue) and ventral iris halves were collected from the right (lentectomized) eyes of the animals. Dorsal iris halves, ventral iris halves, and mature lenses were collected from the left (sham-lentectomized control) eyes. (Only tissues from control eyes containing healthy

intact lenses were used.) The corresponding tissue fragments from each region of the eye were pooled and acetone-dried as described previously (METHODS--section II-D). The lipid-free dry weight of the pooled tissues was measured.

C) *Preparation of Iris/Lens Tissue Homogenates:*

Acetone-dried tissues were homogenized in 1 ml of 0.1 M sodium phosphate (pH 6.5) as previously described (METHODS--section II-D). The homogenates were boiled for 20-30 min and then supplemented with 1 ml of a solution of 0.1 M sodium phosphate/0.01 M EDTA/0.02 M cysteine (pH 6.5).

D) *Preparation of Ethanol-Insoluble GAG-Enriched*

Fraction of Iris/Lens Tissue Homogenates:

The procedures used to isolate ^3H -glucosamine labelled GAGs from tissues include several refinements over the methods previously described for extraction of $^{35}\text{SO}_4$ labelled sulfated GAGs. The reduced negative charge density of hyaluronate polymers in comparison to sulfated GAGs necessitates particular attention to the control of salt and CPC-detergent concentrations in preparative procedures in order to ensure quantitative recovery of hyaluronate. Furthermore, the lower radioactive emission energy of ^3H in comparison to ^{35}S requires additional precautions to minimize quenching in samples prepared for liquid scintillation counting. In the following descriptions, special attention

will be accorded these critical procedural modifications whereas unaltered methods will be outlined only briefly with reference to the detailed descriptions given previously.

Homogenates were subjected to proteolysis with papain for a total of 48 hours at 60°C as described previously (METHODS--section II-E). As before, undigested tissue residues were collected by centrifugation and treated with 0.5 N NaOH for 16-20 hours at 5°C on a rotatory shaker. The alkaline digest was cleared by centrifugation, combined with the supernatant of the prior papain digestion, and TCA was added to a final concentration of 7%. Precipitates formed after 3 h at 0°C were removed by centrifugation (30 min at 10,000 g) and the supernatant was neutralized with NaOH.

After addition of 500 µg of carrier GAG (a mixture of 100 µg of hyaluronic acid, 100 µg chondroitin 4-sulfate, 100 µg chondroitin 6-sulfate, and 200 µg heparin), the TCA-soluble supernatant fraction was transferred to dialysis tubing having an approximate molecular weight cut-off of 1000 daltons. The sample was dialyzed overnight at 5°C against 100-200 volumes of distilled water and then concentrated to a final volume of approximately 2 ml by laying the dialysis bag against a bed of dry polyethylene glycol (Carbowax-PEG 20,000). The concentrated sample was removed, the dialysis bag was rinsed with 1 ml distilled water, and the rinse was combined with the sample. Final

sample volume was adjusted to 3.5 ml with distilled water, after which 11.5 ml of 98% ethanol was gradually added. The formation of ethanol-insoluble GAG precipitates was promoted by the gradual addition of 20-25 drops of saturated aqueous sodium acetate solution. Following overnight incubation at 5°C, the precipitates were collected by centrifugation (45 min; 10,000 g). The pellet was redissolved in 1 ml distilled water and three volumes of 98% ethanol were added. Ethanol-insoluble GAG precipitates were again recovered by addition of a few drops of saturated aqueous sodium acetate, overnight incubation at 5°C, and centrifugation. The supernatant was discarded and the pellet was dried under a vacuum at room temperature. The dried ethanol-insoluble GAG-enriched fraction was stored at -20°C until needed for subsequent analysis as described below.

E) *Measurement of ^3H -Glucosamine Incorporation into Hyaluronate:*

The ethanol-insoluble GAG-enriched fraction from each tissue homogenate was dissolved in 2 ml of 0.05 M sodium acetate/0.1 M NaCl buffer (pH 5.0). The pH of the solution was checked with indicator test paper and, if necessary, adjusted to within the range of pH 5.0-6.0. Two 950 μl aliquots were removed from the sample for treatment as shown in Figure 7. To one aliquot was added 50 μl of 0.05 M sodium acetate/0.1 M NaCl (pH 5.0) containing 10 turbidity reducing units (TRU) of *Streptomyces hyaluroni-*

Figure 7.

Scheme for identifying amount of ^3H -glucosamine label incorporated into hyaluronic acid. The ethanol-insoluble GAG fraction from each iris tissue homogenate was divided into equal aliquots for treatment with either *Streptomyces* hyaluronidase or enzyme buffer alone. In the presence of carrier hyaluronate, all non-degraded GAGs were recovered by CPC precipitation and ethanol precipitation. Label incorporated into hyaluronate was estimated from the difference between counts recovered after hyaluronidase treatment and counts recovered after the mock digestion in buffer alone. Label associated with hyaluronidase-resistant GAGs was estimated from counts recovered after hyaluronidase digestion.

dissolve ethanol-insoluble pellet in
0.05 M acetate buffer containing 0.1 M NaCl (pH 5.0)



divide into equal aliquots



Streptomyces hyaluronidase
digestion

mock enzyme
digestion

add 10 TRU

add buffer only

Streptomyces hyaluronidase



incubate 3 h at 60°C

incubate 3 h at 60°C



boil 15 min

boil 15 min



add 500 µg hyaluronate carrier

add 500 µg hyaluronate carrier



dialyse against 0.1 M NaCl

dialyse against 0.1 M NaCl



precipitate GAGs by addition of 1% cetylpyridinium chloride



dissolve pellet in 2 M NaCNS

dissolve pellet in 2 M NaCNS



recover GAGs by precipitation with ethanol



pellet contains
hyaluronidase-resistant GAGs

pellet contains hyaluronate
plus
hyaluronidase-resistant GAGs

dase. To the second aliquot was added 50 μ l of the same buffer containing no enzyme. Each sample aliquot was incubated 3 hours at 60°C and then boiled 15-20 minutes. Samples were then supplemented with 500 μ g of carrier hyaluronic acid and transferred to dialysis tubing (1000 dalton molecular weight retention). The samples were dialyzed overnight at 5°C against 250 volumes of 0.1 M NaCl. Each sample was removed, the dialysis bag was rinsed with 1 ml 0.1 M NaCl, and the rinse was combined with the sample. With constant agitation, a solution of 1% CPC in 0.1 M NaCl was added dropwise until large flocculent aggregates of CPC-GAG complexes were formed. An additional 5-8 drops of the CPC solution were then added to introduce a slight excess of CPC. (The formation of CPC-GAG complexes by titration in this manner is recommended by Scott [1960] to ensure quantitative recovery of GAGs.) After a 30-minute incubation at room temperature, the precipitates were collected by centrifugation (10,000 g; 45 min; 25°C). The supernatant from each sample was checked for complete removal of GAG by the lack of visible turbidity upon addition of a few drops of 1% CPC in 0.1 M NaCl. To each of the pelleted CPC-GAG fractions was added 0.5 ml 2 M sodium thiocyanate and 100 μ l 98% ethanol. The pellets were solubilized upon heating to 60°C. Three volumes of 98% ethanol were added to each sample and the precipitates which formed at 5°C overnight were collected by centrifugation (10,000 g; 45 min; 5°C). To ensure removal of residual CPC, the

dissolution in 2 M sodium thiocyanate and precipitation with ethanol was repeated once. The final pellet from each sample was dissolved in 2 ml distilled water and added to a vial containing liquid scintillation cocktail (either 10 ml *Phasar* or 15 ml *Aquasol*). Measurements of ^3H radioactivity in each sample were made on a Beckman LS-9000 liquid scintillation counter. Quenching, as monitored by sample channels ratio and H-number methods, was virtually constant in all samples prepared with the same scintillation cocktail. Sample counting efficiency was 34-35% as determined with calibrated $^3\text{H-H}_2\text{O}$ internal standards.

Total ^3H -glucosamine label incorporated into GAGs of all types was determined from the radioactivity recovered from the sample aliquot incubated in the absence of enzyme. The amount of label incorporated into hyaluronate was estimated from the difference between the counts in total GAG and the counts recovered following digestion of the second aliquot with *Streptomyces* hyaluronidase. The amount of label incorporated into GAGs of all other types (i.e., *Streptomyces* hyaluronidase-resistant GAGs) was estimated from the counts recovered following *Streptomyces* hyaluronidase digestion.

IV. Autoradiographic Analysis of $^{35}\text{SO}_4$ Localization in Newt Iris/Lens Tissues

A) *Rationale:*

Autoradiographic methods are routinely employed to demonstrate the locations of β -particle emitting radioisotopes in histological sections of tissues (Rogers, 1967). As an adjunct to the preceding biochemical analysis of $^{35}\text{SO}_4$ uptake into GAGs in the newt iris (METHODS--section II), a preliminary autoradiographic survey was made of the sites of $^{35}\text{SO}_4$ label accumulation in the newt eye. The distribution of incorporated $^{35}\text{SO}_4$ label in autoradiographs is frequently indicative of the locations of sulfated GAG synthesis and deposition in tissues (Dziewiatkowski, 1958).

B) *Preparation of Histological Sections:*

One eye of each experimental animal was lentectomized while the opposite eye was left unoperated. Eighty days after surgery, each newt was given an intraperitoneal injection of 50 μCi of carrier-free $[^{35}\text{S}]\text{H}_2\text{SO}_4$. Four days later, both the lentectomized and unoperated (normal) eyes were excised and fixed in 10% buffered formalin containing 0.5% CPC (METHODS--section I-B). Following a 48-hour fixation period, the tissues were washed in distilled water and then dehydrated through a graded series of ethanol solutions (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and two changes of absolute ethanol; 1-2 hours per solution

except for overnight treatment in 70% ethanol). The dehydrated specimens were then bathed overnight in two changes of Sorvall Methacrylate infiltration solution and embedded in Sorvall Methacrylate embedding medium according to the manufacturer's directions. Polymerization of the methacrylate was conducted under a vacuum at room temperature. Sagittal sections of the eyes were cut at 2 μ m thickness and applied to pre-cleaned glass slides.

C) *Processing for Autoradiography:*

All manipulations were performed under the illumination of a safelight equipped with a Wratten #2 filter and a 15-watt bulb. Slides bearing tissue sections were each dipped for about one second in Kodak NTB-3 emulsion that was kept molten in a 40°C water bath. Excess emulsion was drained onto wet paper towels, and the slides were allowed to dry vertically for 30 minutes at room temperature. The slides were then sealed in black plastic slide-boxes and were exposed in complete darkness for seven days at 5°C. The slides were then developed for two minutes in a solution of one part Kodak Dektol developer and two parts distilled water. The slides were then dipped for 10 seconds in a 1% acetic acid stop-bath, fixed for two minutes in Kodak Rapid Fixer, and rinsed in five changes of distilled water (three minutes each). The slides were allowed to dry at room temperature and were mounted under immersion oil. Light microscopic examination and photography were

performed on a Zeiss Photomicroscope III.

V. Analysis of Chondroitin Sulfate-Degrading Activity
in the Iris during Lens Regeneration

A) *Rationale:*

The actual rate of accumulation of macromolecules within a tissue is regulated both by the gross rate of synthesis of the molecules and by their rate of degradation *in situ*. With respect to the metabolism of GAGs, an altered rate of polysaccharide catabolism might serve as a mechanism by which tissue composition could be modified during developmental processes.

A number of enzymes that can hydrolyze specific linkages in GAGs of the chondroitin sulfate types have been identified in various vertebrate tissues (Dorfman *et al.*, 1972). These enzymes include endoglycosidases that attack internal glycosidic bonds (*e.g.* hyaluronidases) and exoglycosidases that sequentially remove terminal sugar residues (*e.g.* hexosaminidases and glucuronidases). The following procedure was designed to test for the possible presence of endogenous chondroitin sulfate-degrading activity within newt iris tissue and to determine whether this catabolic activity might fluctuate during the process of lens regeneration. The experimental rationale was to assess the ability of cell-free iris tissue extracts to degrade ³H-labelled chondroitin sulfate polymers to low molecular weight products.

B) *Preparation of ^3H -Chondroitin Sulfate*

Substrate (^3H -CSS):

Frozen carcasses were obtained from 30 newts that had been labelled 24 hours with ^3H -glucosamine hydrochloride (10 $\mu\text{Ci}/\text{animal}$). The bodies were thawed, chopped into several pieces, and extracted with two changes of 800 ml acetone. After removing the acetone, the tissues were dried under a vacuum. The dried tissue fragments were transferred to a Sorvall Omni-Mixer and macerated in 200 ml of phosphate buffer (0.1 M sodium phosphate containing 0.1 M NaCl, 0.005 M EDTA, 0.005 M cysteine; pH 6.5).

Following the method of Scott (1960), 2.5 g of crude papain was dissolved in 100 ml of the same phosphate buffer and then pre-activated by incubating 30 min at 60°C. The pre-activated solution was added to the slurry of macerated tissue and the mixture was diluted to a final volume of 500 ml with additional phosphate buffer. Proteolysis was conducted for 24 hours at 60°C. Thereafter, a further 1 g of crude papain (pre-activated in 25 ml buffer) was added to the tissue digest and the proteolytic incubation was continued for 24 hours. The tissue digest was cleared by filtration and TCA was added to a final concentration of 7%. The sediments formed overnight at 0°C were removed by decanting the TCA-soluble fraction. The latter was cleared by centrifugation (900 g; 20 min) and neutralized with concentrated NaOH. Two volumes of 98% ethanol were added and ethanol-insoluble precipitates were allowed to sediment at

5°C overnight.

The clear supernatant was decanted and discarded. The ethanol-insoluble precipitates were collected by centrifugation (900 g; 20 min). The resulting pellet was suspended first in 50 ml of 2 M NaSCN and then in 50 ml of 2 M NaCl. In each case, the suspension was heated at 60°C with occasional shaking, after which the solubilized material was collected as a clear supernatant following centrifugation (10,000 g; 30 min). Material which remained insoluble after both treatments was discarded. The 2 M NaSCN-soluble and 2 M NaCl-soluble supernatants were combined, two volumes ethanol were added, and the ethanol-insoluble precipitates were recovered by centrifugation (10,000 g; 30 min). To remove contaminants from the polysaccharide pellet, a cycle of dissolution in 2 M NaSCN and precipitation with ethanol was repeated several times, using progressively smaller volumes of NaSCN as the solvent. When the precipitates would readily dissolve in 10 ml of 2 M NaSCN, three volumes ethanol were added, and the GAG-enriched fraction was recovered by centrifugation. The pellet was dissolved in 10 ml distilled water and dialyzed against 200 volumes 0.02 M Na_2SO_4 . To the dialyzed fraction was added 1% CPC in 0.02 M Na_2SO_4 until flocculent precipitates were formed. The CPC-GAG precipitates were collected by centrifugation (10,000 g; 45 min; 30°C), washed by suspending in 0.05% CPC/0.02 M Na_2SO_4 solution, and recovered by centrifugation as before. The pellet was dissolved in 2 ml of 2 M NaCl

and 500 μ g carrier heparin was added. Using the method of Roden *et al.* (1972), the various GAGs in the mixture were fractionated on the basis of the differing solubilities of their CPC complexes in solutions of decreasing salt concentration: The solution of CPC-GAG complexes was diluted to a concentration of 1.2 M NaCl by addition of 0.05% CPC solution. The precipitates formed at 37°C were collected as a syrupy floating fraction after centrifugation (10,000 g; 30 min; 40°C). Material recovered in this fraction is characteristically heparin and some heparan sulfate (Roden *et al.*, 1972). The supernatant fraction was diluted further to a final concentration of 0.5 M NaCl. The precipitates formed at 37°C were collected as a pellet following centrifugation (10,000 g; 30 min; 40°C). This fraction was assumed to contain chondroitins 4- and 6-sulfate, dermatan sulfate, and species of heparan sulfate with a low negative charge density (Roden *et al.*, 1972). Hyaluronate characteristically remains in the supernatant fraction in 0.5 M NaCl.

The 0.5 M NaCl-insoluble pellet was dissolved in 2 M NaSCN and the constituent GAGs were recovered by addition of three volumes of ethanol followed by centrifugation (10,000 g; 45 min; 5°C). The pellet was redissolved in 2 M NaSCN and reprecipitated with ethanol to remove residual CPC. To degrade any heparin or heparan sulfates in the GAG fraction, a modification of the nitrous acid hydrolysis method of Lagunoff and Warren (1962) was used: The

ethanol-insoluble GAG pellet was dissolved in 1 ml distilled water and equal volumes of 5% sodium nitrite and 33% acetic acid were added. After shaking, the mixture was incubated 2 h at room temperature. The reaction was stopped by addition of NaOH to neutrality and the digest was dialyzed against 500 volumes of 0.5 M NaCl. Non-degraded GAGs were precipitated by dropwise addition of 1% CPC/0.5 M NaCl and collected by centrifugation (10,000 g; 45 min; 35°C). The CPC-GAG complexes were dissociated in 1 ml of 2 M NaSCN and the GAGs were recovered by addition of three volumes ethanol and centrifugation (10,000 g; 45 min; 5°C). This procedure was repeated once to remove residual CPC from the polysaccharide pellet. The ethanol-insoluble pellet was dissolved in 1 ml distilled water, three volumes ethanol were added, and the GAG was precipitated by addition of a few drops of saturated aqueous sodium acetate solution. The pellet was recovered by centrifugation (10,000 g; 45 min; 5°C), dried under a vacuum, and then dissolved in 1.5 ml distilled water. This solution was used as the ^3H -labelled chondroitin sulfate substrate (^3H -CSS) for assays of chondroitin sulfate-degrading activity in iris tissue extracts. The specific activity of the ^3H -CSS preparation was approximately 9600 cpm/mg GAG as determined by a modified carbazole method for uronic acid (Davidson, 1966), using chondroitin 4-sulfate (commercial preparation) as a standard.

In order to characterize the macromolecular distribu-

tion of the ^3H -label in the ^3H -CSS solution, aliquots of 20 μl (approximately 700 cpm) were removed for treatment with chondroitinase AC, chondroitinase ABC, and nitrous acid as follows:

[1] Chondroitinase AC treatment:

A 20 μl aliquot of ^3H -CSS was incubated 3 h at 37°C in the presence of 150 μl 1:5 diluted Tris buffer (pH 7.3) and 0.5 unit chondroitinase AC. Following incubation the sample was boiled 10 min.

As a control (mock digestion) a second 20 μl of ^3H -CSS was treated as above, but in buffer solution only (no enzyme).

Following treatment, each sample was supplemented with carrier GAG (250 μg each of chondroitin 4-sulfate and chondroitin 6-sulfate) and then dialyzed against 400 volumes 0.1 M NaCl. Non-degraded GAG was recovered by CPC-precipitation, dissolution in 2 M NaSCN, and ethanol precipitation as described previously. The ethanol-insoluble GAG pellet was dissolved in 2 ml distilled water and added to 10 ml Aquasol cocktail for liquid scintillation counting. The proportion of ^3H -label bound in chondroitins 4- and 6-sulfate (C4S/C6S) was determined from the difference between the counts recovered in the control sample and the counts recovered in the chondroitinase AC treated sample.

[2] Chondroitinase ABC treatment:

Chondroitinase ABC digestion and mock digestion were performed in the same manner as described for chondroitinase AC treatment, with the exception that 0.5 unit chondroitinase ABC was used and that all buffers were at pH 8.0.

Non-degraded GAG was collected after dialysis as described above. The proportion of ^3H -label in dermatan sulfate (DS) was calculated from the difference between the counts recovered after chondroitinase ABC treatment and the counts recovered after chondroitinase AC treatment.

[3] Nitrous acid treatment:

To a vial containing 100 μl distilled water was added 20 μl ^3H -CSS, 120 μl 5% sodium nitrite, and 120 μl 33% acetic acid. The mixture was incubated 90 min at room temperature after which the reaction was stopped by adding 140 μl 5 N NaOH and 500 μl 0.2 M sodium phosphate (pH 7.0).

As a control (mock digestion), 120 μl 5% sodium nitrite, 120 μl 33% acetic acid, 140 μl 5 N NaOH and 500 μl 0.2 M sodium phosphate (pH 7.0) were combined and added to a vial containing 100 μl distilled water and 20 μl ^3H -CSS. The mixture was incubated 90 min at room temperature.

After dialysis and recovery of non-degraded GAGs as described above, the percentage of ^3H label in heparin and heparan sulfate (Hep/HS) was estimated from the proportion of counts removed in the nitrous acid treated sample in comparison with the control sample.

[4] Sequential chondroitinase ABC/

nitrous acid treatment:

Separate 20 μ l aliquots of ^3H -CSS were subjected to chondroitinase ABC digestion and mock enzyme digestion as described above.

To the enzyme treated sample was added 170 μ l each of 5% sodium nitrite and 33% acetic acid. After 90 min at room temperature, 198 μ l 5 N NaOH and 400 μ l 0.2 M sodium phosphate (pH 7.0) were added.

To the mock digested control sample was added a mixture of 170 μ l 5% sodium nitrite, 170 μ l 33% acetic acid, 198 μ l 5 N NaOH and 400 μ l 0.2 M sodium phosphate. The mixture was incubated 90 min at room temperature.

Non-degraded GAG was recovered after dialysis as described above. The percentage of ^3H -label not incorporated into GAGs of the C4S/C6S, DS, or Hep/HS types was determined from the proportion of counts recovered in the chondroitinase/nitrous acid treated sample in comparison with the total counts recovered in the control sample.

C) *Determination of ^3H -CSS Degrading*

Activity in Iris Tissue Extract:

For each determination 20 newts were bilaterally lens-tectomized as described previously (METHODS--section I-A) and then reared to a desired stage of lens regeneration (5, 10, 15, 20, or 30 days). To compare the condition of the normal untraumatized iris, some determinations were per-

formed on groups of 20 unoperated animals. Whole iris rings were excised from each group of animals, as previously described (METHODS--section II-C). Newly regenerated lens tissue was included with the iris tissue excised from regenerating eyes, but the mature lenses of normal eyes were processed separately. Excised tissues were rinsed in Amphibian Ringer (pH 7.55), transferred to a glass homogenizer, and washed with two 1 ml changes of a 0.1 M sodium formate/0.15 M NaCl buffer (pH 3.7) supplemented with Gentamicin (25 μ g/ml). While immersed in an ice-water bath, the tissue was homogenized in 1 ml of the same formate buffer using a hand-held teflon pestle. The homogenate was cleared by centrifugation (8000 g; 5 min) after which 100 μ l was withdrawn for protein determination by the micro-method of Bradford (1976) [Biorad].

To a 400 μ l aliquot of the remaining iris tissue extract was added 50 μ l of ^{3} H-CSS solution. As a control, a second 400 μ l aliquot of the extract was immediately boiled for 10 min, after which 50 μ l ^{3} H-CSS was added. Both samples were shaken and then incubated 24 h at room temperature. Following incubation each sample was boiled 10 min. After addition of 500 μ g carrier GAG (250 μ g each of chondroitins 4- and 6-sulfate), the samples were dialyzed overnight against 1000 volumes of 0.1 M NaCl using dialysis tubing with a molecular weight cut-off of approximately 1000 daltons. Non-degraded GAG was precipitated by dropwise addition of 1% CPC in 0.1 M NaCl and collected by



centrifugation (10,000 g; 45 min; 35°C). The pellets were dissolved in 0.5 ml 2 M NaSCN and GAG was recovered by addition of three volumes ethanol. This procedure was repeated once to remove residual CPC. The ethanol-insoluble pellet from each sample was dissolved in 2 ml distilled water and added to 15 ml *Aquasol* cocktail for liquid scintillation counting. Measurements were made on a Beckman LS-9000 counter. Quenching was nearly constant from sample to sample as monitored by sample channels ratio and $H^{\#}$ methods. Counting efficiency for ^3H was approximately 34% as determined with calibrated $^3H-H_2O$ internal standards.

The chondroitin sulfate-degrading activity in each iris tissue extract was estimated from the reduction in ^3H -activity in the test sample relative to total activity in the boiled control sample.



VI. Analysis of Hyaluronidase

Activity in the Iris

A) *Rationale:*

Among the most widely distributed agents of glycosaminoglycan catabolism in animal tissues are the endoglycosidases collectively termed the *hyaluronidases*. With the exception of the hyaluronidase from leech salivary gland, all the known hyaluronidases of animal origin operate by hydrolyzing the internal $\beta 1 \rightarrow 4$ glycosidic bonds that join the N-acetylhexosamine and glucuronic acid groups in polymers of hyaluronate, chondroitin, and chondroitin sulfate (Meyer, 1971). Hyaluronidases have been purified from the mammalian testis (Borders and Raftery, 1968), the canine submandibular gland (Tan and Bowness, 1968), and rat liver lysosomes (Aronson and Davidson, 1967a). Hyaluronidase activities have also been detected in numerous other tissues including skin and spleen (Meyer *et al.*, 1941), kidney, lung, and blood serum (Bollet *et al.*, 1963), and the venoms of bees and some snakes (Barker *et al.*, 1963; Chain and Duthie, 1940). In several developmental systems, a transient increase in tissue hyaluronidase activity is temporally correlated with the cessation of both the proliferation and migration of mesenchymal cells and the onset of cell differentiation (Toole and Gross, 1971; Toole and Trelstad, 1971; Toole, 1972; Toole, 1981).

The following protocol was employed to test for the

presence of an endogenous hyaluronidase within the iris. The analytical approach was to incubate substrates of purified hyaluronate with iris tissue extracts and to assess the extent of resulting polymer hydrolysis. The latter was quantified using a colorimetric method (Reissig *et al.*, 1955) for determination of the N-acetylhexosamine end-groups liberated through cleavage of internal β 1 $\rightarrow$ 4 hexosaminidic bonds. Similar methods have been used successfully by previous authors to demonstrate hyaluronidase activities in a variety of tissues (Bollet *et al.*, 1963; Aronson and Davidson, 1967b; Toole and Trelstad, 1971; Toole and Gross, 1971; Toole, 1972; Polansky *et al.*, 1974; Orkin and Toole, 1980).

B) *Preparation of Iris Tissue*

Extract-Substrate Reaction Mixtures:

Groups of 20-30 bilaterally lentectomized newts were reared to various regeneration stages as described previously (METHODS--section I-A). To provide normal iris tissues, some experiments were performed using groups of unoperated animals. Whole iris rings were removed from each group of newts and the tissues were pooled in ice-cold sterile Amphibian Ringer (pH 7.55) supplemented with antibiotic (50 μ g/ml Gentamicin). The irises from lentectomized eyes included any newly regenerated lens tissue present at the dorsal margin. Normal iris tissues did not include lens tissue. The irises were transferred to a glass homo-

genizer and washed with two 0.5 ml changes of formate buffer (0.1 M sodium formate containing 0.15 M NaCl and 10 µg/ml Gentamicin, pH 3.7). The rinse buffer was removed and 100 µl of fresh formate buffer was added per 10 iris rings in the pooled tissues (e.g. 600 µl added to 60 whole iris rings collected from 30 animals). With the homogenizer immersed in an ice-water bath, the tissues were homogenized using a hand-held pestle. The homogenate was cleared by centrifugation (5 minutes at 7000 g and 5°C in a Beckman Microfuge) and the supernatant was collected. Several 100 µl aliquots were removed from the supernatant and transferred to small stoppered plastic vials. The remaining extract was boiled 5 minutes and retained for subsequent determination of protein content by the micro-method of Bradford (1976) [Biorad], using bovine serum albumin as a reference standard.

To serve as a heat-inactivated control, one vial of the iris tissue extract was immediately boiled for 5-10 minutes. To each vial, including the control, was then added one of the following substrate solutions:

- [1] 300 µg hyaluronic acid (commercial preparation from human umbilical cord) in 50 µl enriched formate buffer
- [2] 300 µg hyaluronic acid in 50 µl formate buffer containing 7.5 mM D-saccharic acid 1,4-lactone (an inhibitor of β -glucuronidase activity; Levvy and Marsh, 1959)
- [3] 300 µg of chondroitin 6-sulfate (commercial preparation from shark cartilage) in 50 µl formate buffer.

Only substrate solution [2] was used for heat-inactivated control samples. Any of solutions [1], [2], or [3] were used in the test vials.

After addition of substrate, the reaction mixtures were incubated at room temperature for various time periods. Thereafter, each vial was boiled 5-10 minutes to deactivate any enzyme activities.

C) *Determination of N-acetylhexosamine*

End-Group Release:

The quantity of N-acetylhexosamine reducing end-groups exposed through cleavage of the hexosaminidic bonds in the substrate polymers was determined by the method of Reissig *et al.* (1955). The standard method was modified by proportionately reducing sample volume and all reagent volumes:

Stock solutions were prepared of tetraborate reagent (0.2 M potassium tetraborate, pH 9.4) and DMAB reagent (10 g p-dimethylaminobenzaldehyde in a solution of 87.5 ml glacial acetic acid and 12.5 ml 10 N HCl). The stock DMAB solution was stored in the dark at 5°C for up to one month and was diluted with nine volumes glacial acetic acid immediately before use.

To each iris extract-substrate reaction mixture (150 μ l) was added 30 μ l of tetraborate reagent. The mixture was heated exactly three minutes in a boiling water bath and then cooled in tap water. A 900 μ l volume of diluted DMAB reagent was added. The mixture was incubated

for 20 minutes at 37°C and cooled in tap water. Any turbidity present was removed by centrifugation (7000 g; 2 min). (The latter was found to be necessary for determination of the heat-inactivated control samples in which precipitates of undegraded substrate polymers were formed upon addition of the acidic DMAB reagent.) The supernatant was collected and its absorbance at 585 nm was measured under the visible light source of a Beckman DU-8 spectrophotometer. The quantity of N-acetylglucosamine reducing end-groups liberated from hyaluronate polymers was determined from a standard curve of absorbance *versus* known concentrations of N-acetyl-D-glucosamine (commercial preparation). For estimation of N-acetylgalactosamine released from chondroitin 6-sulfate substrate, the standard curve was prepared using N-acetylgalactosamine (commercial preparation) for the reference standards.

D) *Determination of pH Optimum of
Iris Tissue Hyaluronidase Activity:*

It was necessary to modify the methods described above in order to determine the pH optimum of the hyaluronidase activity in iris tissue extracts. In each experiment, whole iris rings were harvested from 60 normal non-lentectomized newts. The pooled iris tissues were homogenized in 600 μ l of 0.1 M sodium formate/0.15 M NaCl (pH 3.7) as described above (METHODS--section VI-B). The homogenate was cleared by centrifugation (7000 g; 5 min; 5°C) and the

supernatant fraction was dialyzed overnight against 500 ml of 0.15 M NaCl. The dialyzed iris extract was then divided into 50 μ l aliquots that were each transferred to a small plastic vial. To each vial was added 50 μ l of 0.3 M NaCl containing 200 μ g hyaluronate substrate and 7.5 mM saccharic acid 1,4-lactone. Each of the vials was supplemented with 50 μ l of 0.3 M sodium formate or sodium phosphate buffer at a different pH. One experiment was conducted using 0.3 M sodium formate buffer solutions (at pH 2.5, 3.0, 3.5, 3.7, 4.0, 4.5, 5.0, 5.5, and 6.0). Another experiment was conducted using 0.3 M sodium phosphate buffers (at pH 5.5, 6.0, 6.5, 7.0, 7.5). The iris extract-substrate reaction mixtures were all incubated 6 h at room temperature after which the samples were boiled for five minutes to stop the reaction. In each experiment an appropriate control was included in which an aliquot of the iris extract was heat-inactivated by boiling prior to incubation with the substrate solution.

The N-acetylhexosamine end-groups released in each reaction mixture were determined by the method of Reissig *et al.* (1955) as described above (METHODS--section VI-C) with slight modifications. In order to make the assay less sensitive to differences in sample pH, a solution of 0.5 M potassium tetraborate was used as the tetraborate reagent. Due to the larger sample volume (150 μ l), the volumes of reagents used were increased to 50 μ l for the 0.5 M tetraborate reagent and 1000 μ l for the diluted DMAB reagent.

Standard curves of absorbance *versus* N-acetyl-D-glucosamine standards of known concentration were determined for conditions of differing pH in order to compensate for a slight pH-dependent variation in colour yield in the modified assay procedure.

E) *Assay for Microbial Hyaluronidase*

Activity in Iris Tissue Extract:

Since a variety of microorganisms are known to produce hyaluronidases (Meyer, 1971), it was necessary to consider the possibility that degradation of hyaluronate substrates within iris tissue extracts might result from microbial contamination rather than from an iris-derived hyaluronidase. The hyaluronate digestion products of both animal tissue hyaluronidases and microbial hyaluronidases bear reducing-terminal N-acetylhexosamine groups and therefore undergo similar colour reactions in the assay procedure of Reissig *et al.* (1955). The oligosaccharides released by microbial hyaluronidases differ, however, from those liberated by hyaluronidases of animal origin in that the former bear Δ -4,5-unsaturated glucuronide residues at their non-reducing ends, whereas the latter contain only saturated glucuronic acid groups (Meyer, 1971). Since the unsaturated uronides have a characteristic light absorption peak at 235 nm (Linker, 1966), the products of microbial hyaluronidase action can be readily detected by spectrophotometry.

Iris tissue extract in 400 μ l formate buffer (pH 3.7)

was prepared from the whole irises of 20 normal non-lentectomized newts as described above (METHODS--section VI-B). Three 100 μ l aliquots were removed. (The remaining extract was retained for protein determination as already described.) One aliquot was immediately boiled for five minutes to serve as a heat-inactivated control sample. Substrate solution (300 μ g hyaluronate in 50 μ l formate buffer containing 7.5 mM saccharic acid 1,4-lactone) was then added to each sample, including the control. The control and test samples were incubated 18 hours at room temperature and then boiled for five minutes. One of the test samples was used for measurement of N-acetylhexosamine release by the method described above (METHODS--section VI-C). The control sample and the other test sample were used for detection of unsaturated uronides as described below.

To ensure that the experimental methods were adequate for measurement of unsaturated uronide formation, reaction mixtures were also prepared using a purified hyaluronidase of microbial origin, *Streptomyces* hyaluronidase. This enzyme is known to degrade hyaluronate polymers to tetrasaccharide and oligosaccharide fragments that bear the characteristic Δ -4,5-unsaturated glucuronide groups at their non-reducing ends (Ohya and Kaneko, 1970). One reaction mixture was prepared that contained 300 μ g of hyaluronate substrate and 10 turbidity reducing units (TRU) of *Streptomyces* hyaluronidase in a final volume of 150 μ l of 0.05 M sodium acetate buffer (pH 5.0) containing 0.1 M NaCl. A

control sample was prepared in the same manner, but using heat-inactivated *Streptomyces* hyaluronidase. Both the control and test samples were incubated 18 hours at 60°C. Thereafter, the samples were boiled for 10 minutes.

The method of Linker (1966) was followed for detection of unsaturated uronide release, with the exception that sample volume and all reagent volumes were proportionately reduced: To each sample reaction mixture (150 μ l) was added 300 μ l distilled water and 500 μ l of 6% perchloric acid. After thorough mixing, the sample was cleared by centrifugation (15 minutes at 900 g). The absorbance of each sample at 235 nm was measured under the ultraviolet light source of a Beckman DU-8 spectrophotometer. The locations of absorbance peaks in the test samples were determined by wavelength scanning, using the appropriate heat-inactivated control samples as the reagent blanks.

RESULTS

I. Incorporation of $^{35}\text{SO}_4$ into Sulfated GAGs within the Newt Iris during Lens Regeneration

A) *Stage-Dependent Variation in the Total Rate of $^{35}\text{SO}_4$ Uptake into Sulfated GAGs:*

To serve as an index of change in the net rate of sulfated GAG synthesis within the iris during lens regeneration, the total amounts of $^{35}\text{SO}_4$ label incorporated into GAGs were compared in the normal irises of non-lentectomized newts and in the regenerating iris tissues from animals at various intervals after lentectomy.

Groups of animals were lentectomized and reared to a series of different regeneration stages. As controls representing the normal condition of the untraumatized iris, other groups of animals were left unoperated. The animals were administered [^{35}S]H₂SO₄ and, after a suitable time interval (usually 48 hours), whole iris rings were excised. The irises from newts at the various regeneration stages were collected so as to include any newly formed lens tissue present at their dorsal margins. In contrast, the normal irises of non-lentectomized animals were collected separately from the large mature lenses of the normal eyes. Corresponding tissues were pooled and homogenized and total GAG-bound radioactivity was measured in the ethanol-

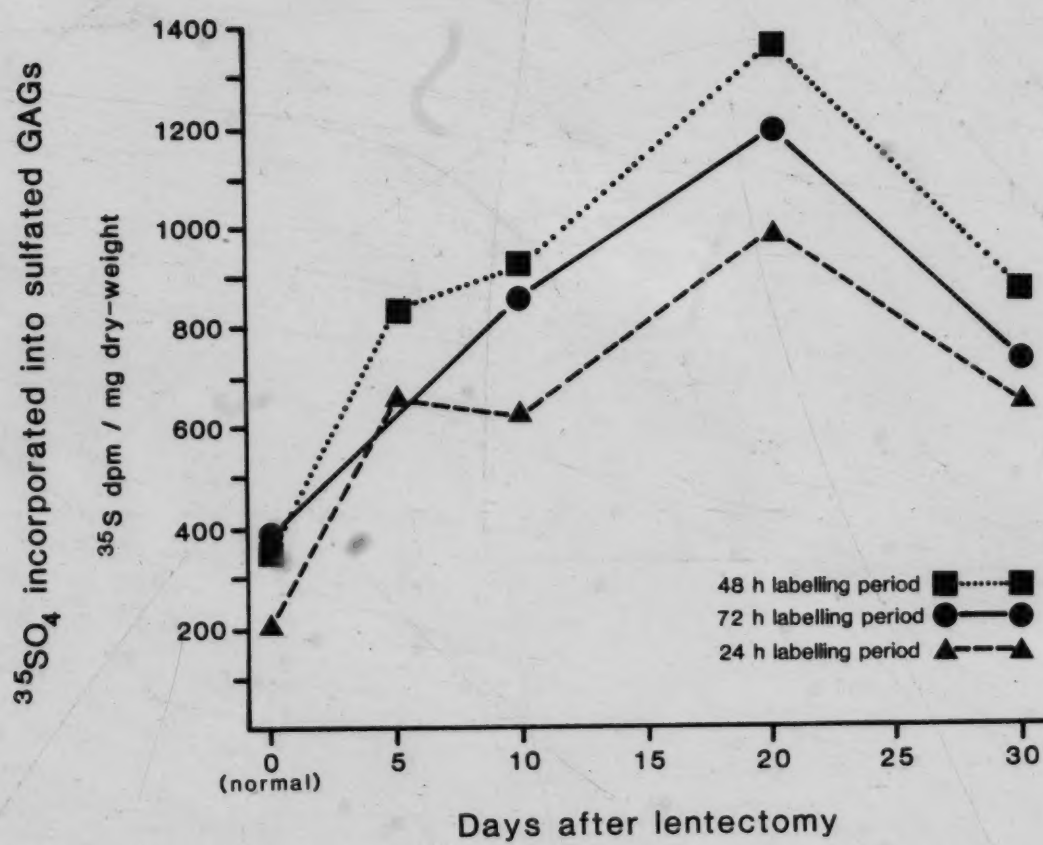
insoluble, CPC-precipitable material extracted from papain digests of the ground tissues.

In preliminary experiments, labelling periods of 24, 48, and 72 hours were compared in order to establish optimal conditions for incorporation of the $^{35}\text{SO}_4$ label into iris tissues at the regeneration stages under examination. In these trials, tissue homogenates were prepared from the whole irises of groups of 10 to 15 animals at each stage of regeneration. As illustrated in Figure 8, a labelling period of 48 hours following administration of $^{35}\text{SO}_4$ to the experimental animals yielded the maximum incorporation of radioactive isotope into iris tissue GAGs at all the regeneration stages examined (5, 10, 20, and 30 days after lenticectomy). In addition, incorporation of the labelled precursor into the normal iris tissues of unoperated animals was only negligibly lower at 48 hours after isotope administration than at 72 hours. A precursor incorporation period of 24 hours provided the least efficient labelling of GAGs within iris tissue in both the lenticotomized and unoperated groups of animals.

Regardless of the duration of the precursor labelling period, qualitatively similar changes were observed in the rates of $^{35}\text{SO}_4$ uptake into GAGs within the iris at different stages of lens regeneration (Figure 8). Labelling was consistently lowest within the normal iris tissues from non-lenticotomized eyes. Following lenticectomy, the iris tissues accumulated two to three-fold greater amounts of

Figure 8.

Comparison of the amounts of $^{35}\text{SO}_4$ uptake into the sulfated GAGs of iris tissue after 24, 48, and 72-hour labelling periods at five stages of lens regeneration. Data points represent values from single experiments based on homogenates of 20-30 pooled whole irises.



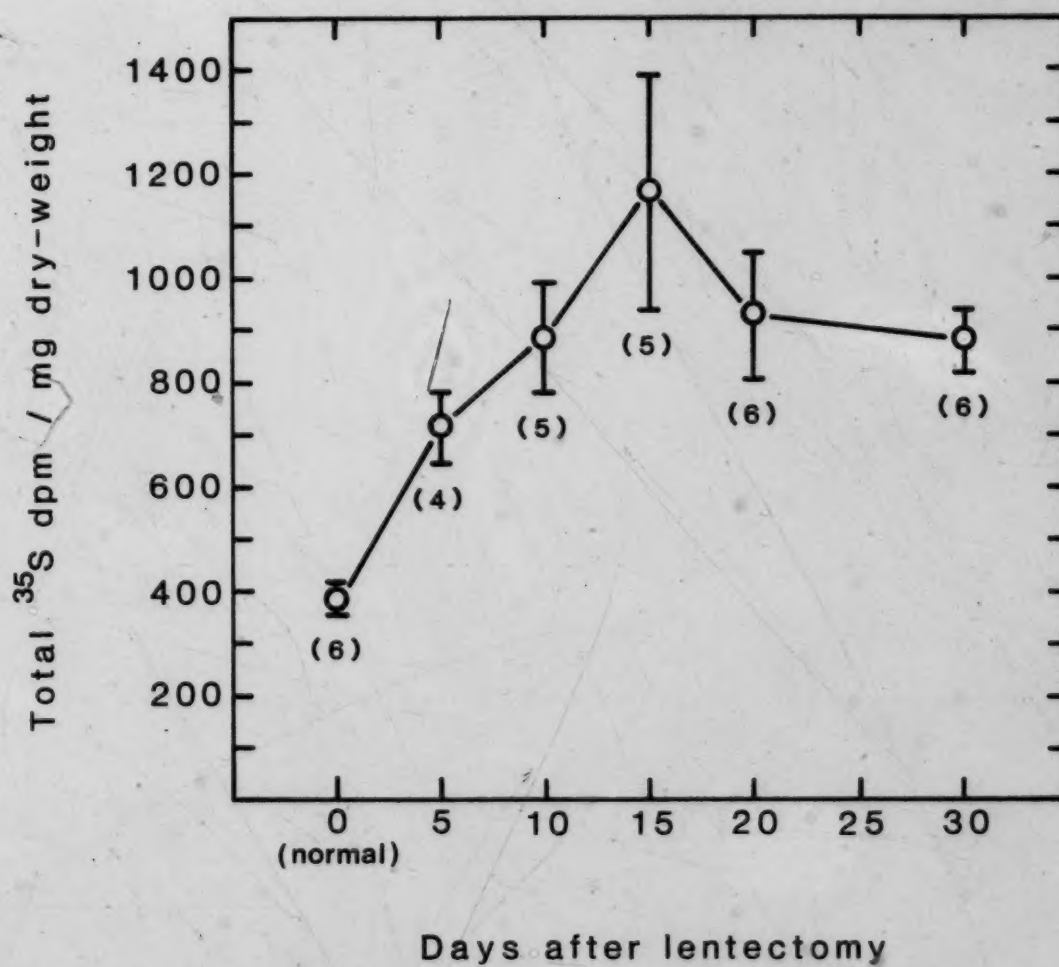
$^{35}\text{SO}_4$ into GAG in comparison with the normal irises. Of the regeneration stages examined, maximum labelling occurred during the 24 to 72-hour period that immediately preceded day 20 after lentectomy.

On the basis of these preliminary trials, a $^{35}\text{SO}_4$ labelling period of 48 hours was selected for use in all subsequent experiments since it yielded the highest levels of isotope incorporation in irises of lentectomized animals as well as near maximal incorporation in normal iris tissues. Determinations were made on iris tissues from groups of 25 to 30 newts; four to six replicate experiments were performed at each regeneration stage examined. The total amounts of $^{35}\text{SO}_4$ label incorporated into GAGs were compared in the whole irises of normal, non-lentectomized newts and in irises of animals at five intervals after lentectomy (5, 10, 15, 20, and 30 days).

Uptake of $^{35}\text{SO}_4$ into the GAGs of iris tissue was markedly higher at all stages of regeneration than in the normal untraumatized iris (Figure 9). Incorporation of label was elevated nearly two-fold by five days after lentectomy, the earliest stage of regeneration examined, and was significantly higher than normal by day 10 ($P \leq 0.05$; analysis of variance--Duncan's multiple range test). There was considerable variation in the rates of labelling of iris tissues from separate groups of experimental animals at regeneration stages of 10, 15, and 20 days. Presumably, this was due to the variability in the inherent rates of regen-

Figure 9.

Incorporation of $^{35}\text{SO}_4$ label into the sulfated GAGs of whole irises from normal, non-lentectomized newts (day 0) and newts at five intervals after bilateral lentectomy. Data points represent mean values from a number of replicate experiments (indicated in parentheses), each based on a homogenate of 50-60 pooled irises. Bars represent $\pm$ standard error of the mean.



eration within individual newts, as has been previously reported (T. Yamada, 1967; Connelly, 1978). Despite this variability, however, the mean rate of incorporation of $^{35}\text{SO}_4$ into GAGs reached a peak at 15 days after lentectomy. At this point uptake of label was elevated approximately three-fold over that in the normal iris ($P \leq 0.01$) and was significantly higher than at day 5 of regeneration. The rate of labelling declined slightly by 20-30 days after lentectomy, but remained significantly enhanced ($P \leq 0.05$) in comparison with the normal iris. Collectively these data indicate that lentectomy triggered a transient increase in the rate of precursor incorporation into newly synthesized sulfated GAGs within the tissue of the newt iris.

B) Distribution of $^{35}\text{SO}_4$ Label among Different

Types of Sulfated GAG:

The different types of iridal GAGs incorporating the $^{35}\text{SO}_4$ label during lens regeneration were identified by the scheme described in Figure 6 (see METHODS--section II-G). Aliquots of the ethanol insoluble GAG fraction from homogenates of whole iris tissues were digested with chondroitinase AC, chondroitinase ABC, nitrous acid, or a combination of chondroitinase ABC and nitrous acid. After each treatment, non-degraded GAGs were recovered by CPC-precipitation. The radioactivity in each fraction was expressed as a percentage of the label present in a control aliquot

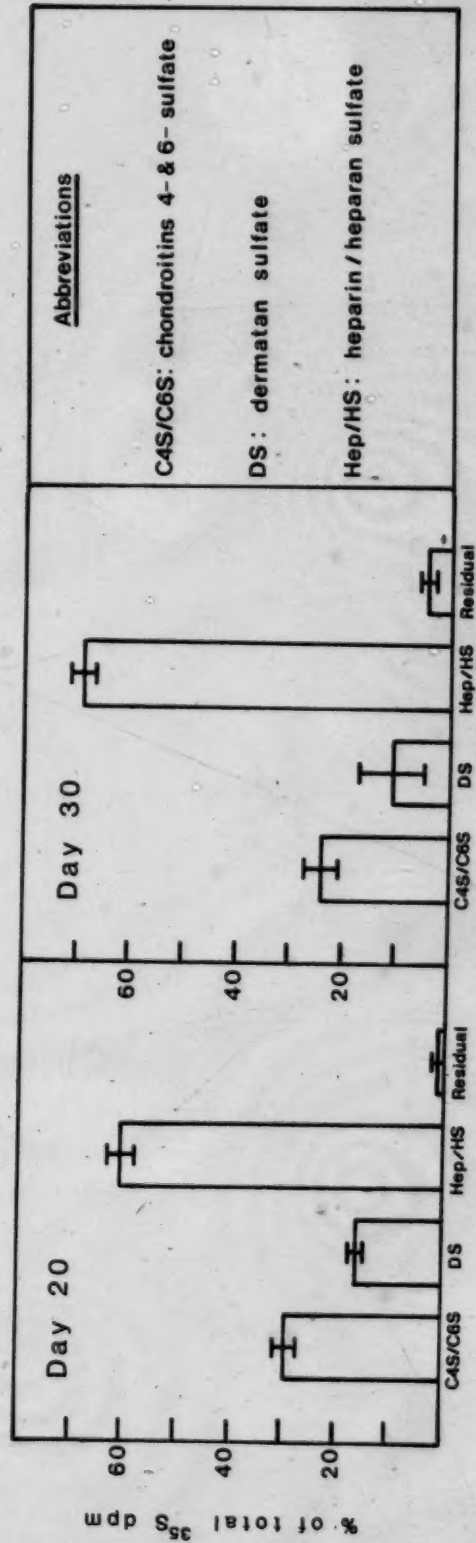
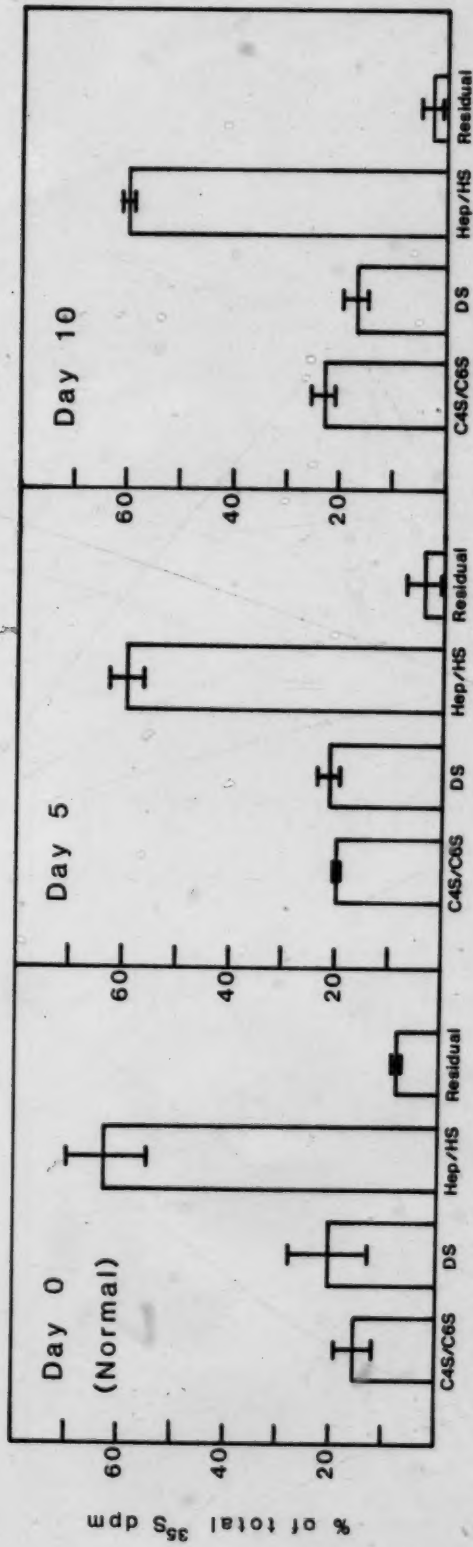
treated with a mock digestion in buffer alone. The differences in the radioactivity remaining after each degradative treatment were used to resolve the relative proportions of total label residing in each of three classes of sulfated GAG: the chondroitins 4- and 6-sulfate (C4S/C6S), dermatan sulfate (DS), and heparin/heparan sulfate (Hep/HS).

Profiles of $^{35}\text{SO}_4$ uptake by these different types of GAG, as determined for normal irises and those at four stages of regeneration, are shown in Figure 10. Duplicate or triplicate determinations were performed at each stage using separate groups of 25-30 newts.

In contrast to the dramatic elevation in the rate of $^{35}\text{SO}_4$ uptake into iridal GAGs which followed lentectomy, the relative distribution of the label among different varieties of GAG remained similar in normal irises and in regenerating irises of all stages. In each case approximately 60-70% of the total GAG-bound radioactivity was sensitive to nitrous acid degradation and resistant to chondroitinase ABC digestion, indicating that Hep/HS accounted for the majority of label uptake. A comparison of the ratios of $^{35}\text{SO}_4$ label sensitive to chondroitinase AC and chondroitinase ABC treatments indicated that C4S/C6S and DS accounted for 16-29% and 11-22% of total $^{35}\text{SO}_4$, respectively. A sequential treatment with both chondroitinase ABC and nitrous acid should, in principle, degrade all GAGs of the C4S/C6S, DS, and Hep/HS types and was indeed successful in removing all but a minor portion (1-9%) of the total label

Figure 10.

Relative amounts of $^{35}\text{SO}_4$ incorporated within three classes of sulfated GAG. Profiles are presented for normal irises (day 0) and irises at four stages of lens regeneration. Histograms indicate the percent of total GAG-bound label associated with each sulfated GAG type and represent mean values of replicate experiments using homogenates of 50-60 pooled irises. Three replicate experiments were performed at day 10 and day 30 regeneration stages. At all other stages two determinations were made. Error bars represent $\pm$ standard error of the mean.



at each stage examined. The identity of this small residual fraction could not be determined by our methods.

These data indicate that throughout lens regeneration a stable relationship is maintained with respect to the relative rates of $^{35}\text{SO}_4$ incorporation into newly synthesized GAGs of the chondroitin sulfate, dermatan sulfate, and heparin/heparan sulfate types.

C) *Relative Proportions of $^{35}\text{SO}_4$ Label in Dorsal Iris, Ventral Iris, and Lens Tissues:*

To determine whether the incorporation of $^{35}\text{SO}_4$ into GAGs of the normal and regenerating iris was restricted to regions with lens-forming capacity, the relative amounts of total label incorporated into dorsal and ventral halves of the iris were compared. Labelling within the mature lenses of normal non-lentectomized animals and within the newly formed lenses of one group of 30-day regenerate animals was also examined. At all other stages of regeneration the nascent lens vesicles were too small to allow their independent excision. Therefore lens tissues at regeneration stages of 5, 10, and 20 days were included with dorsal iris halves in the preparation of tissue homogenates.

Determinations were performed on groups of 25-30 newts from which dorsal iris, ventral iris, and lens tissues (where applicable) were excised and homogenized separately. Radioactivity was measured in the ethanol-insoluble, CPC-precipitable fractions from papain digests of the tissue

homogenates.

Data from these experiments are shown in Table 3. In both normal and regenerating irises, the relative amounts of GAG-bound $^{35}\text{SO}_4$ incorporated into dorsal and ventral iris halves were not markedly different (44-55% and 38-51%, respectively). In the case of normal animals and advanced (30 day) regenerates, only a minor portion of total $^{35}\text{SO}_4$ label was present in lens tissues. These results indicate that throughout the process of lens regeneration, uptake of $^{35}\text{SO}_4$ label into newly synthesized GAGs occurs predominantly within iris tissue proper and to similar extents in both lens-forming (dorsal) and non-lens-forming (ventral) regions. Comparatively little label is accumulated into GAGs within either mature or nascent lens tissues.

Table 3. Relative amounts of $^{35}\text{SO}_4$ in GAG of dorsal iris, ventral iris, and lens

Regeneration stage	Dorsal iris		Ventral iris		Lens	
	^{35}S -dpm	(% of total)	^{35}S -dpm	(% of total)	^{35}S -dpm	(% of total)
Normal	1,351	(50%)	1,169	(44%)	148	(6%)
Normal	1,198	(48%)	1,129	(46%)	156	(6%)
Normal	1,017	(53%)	730	(38%)	187	(9%)
5 day	1,886	(55%)	1,519	(45%)	N.D.	
10 day	2,526	(55%)	2,063	(45%)	N.D.	
20 day	3,078	(51%)	2,953	(49%)	N.D.	
30 day	2,099	(44%)	2,482	(51%)	223	(5%)
30 day	2,546	(52%)	2,336	(48%)	N.D.	
30 day	2,594	(52%)	2,350	(48%)	N.D.	

N.D.: Indicates that values were not determined for isolated lens tissue. In these cases regenerated lens tissues were included with dorsal iris halves.

II. Incorporation of ^3H -Glucosamine into Hyaluronate
and *Streptomyces* Hyaluronidase-Resistant GAGs
within the Iris during Lens Regeneration

In a series of experiments, the uptake of ^3H -glucosamine label into *Streptomyces* hyaluronidase-sensitive GAG and hyaluronidase-resistant GAGs was measured in newt iris tissues at various stages of lens regeneration. The objectives of this study were to detect any stage-dependent fluctuations in the rate of synthesis of the non-sulfated GAG, hyaluronate, during lens regeneration and to determine whether this activity differed in the dorsal (lens-forming) and ventral (non-lens-forming) regions of the iris. These experiments were also used to provide supplementary information regarding the patterns of precursor uptake into sulfated GAGs within the iris following lentectomy.

Between 50 and 60 animals were used in each experiment. One eye of each animal was lentectomized and, as a contralateral control, the opposite eye was sham-lentectomized by incising the cornea while leaving the lens *in situ*. The animals were reared for one of various intervals after surgery and then administered ^3H -glucosamine intraperitoneally. After 24 hours of labelled precursor uptake, dorsal and ventral iris halves were removed from both lentectomized and sham-lentectomized (control) eyes. The dorsal iris halves of lentectomized eyes were collected so as to include any newly-formed lens tissue present at the iris

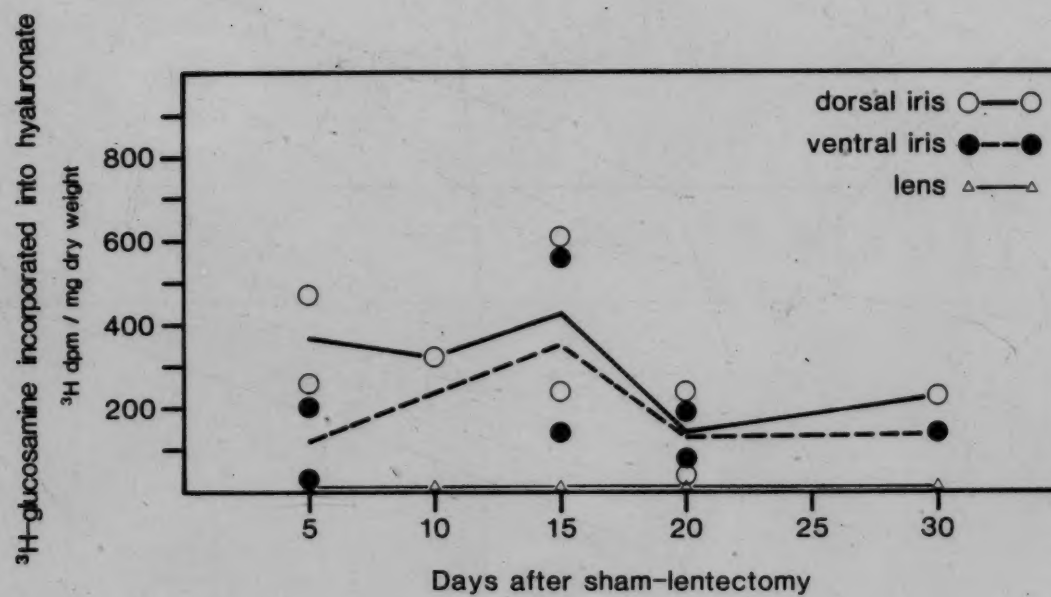
margin. In control eyes the iris tissues and mature lenses were collected separately. Corresponding tissues were pooled and homogenized. Radioactivity was measured in the ethanol-insoluble, CPC-precipitable material from the papain digest of each homogenate. Label incorporated into hyaluronate was identified by its sensitivity to digestion with *Streptomyces* hyaluronidase, an enzyme that specifically degrades hyaluronic acid polymers (see METHODS--section III-E). The hyaluronidase-resistant label was used as a measure of combined precursor uptake into the GAGs of all other classes (presumably consisting largely of sulfated GAG types, with a possible component of unsulfated chondroitin polymers).

A) Labelling of Hyaluronate:

Within the non-regenerating control iris tissues of sham-lentectomized eyes, the amounts of ^3H -label that were incorporated into hyaluronate were consistently low--ranging from 20 to 600 dpm per mg of tissue dry weight (Figure 11). Although there was considerable variability between the values obtained in replicate experiments, labelling of hyaluronate within the dorsal halves of the control iris tissues tended to be slightly greater during the first 15 days after sham-lentectomy than after 20 to 30 days. Within the ventral iris region, labelling of hyaluronate was similar at most times after surgery, although a considerably higher than average level of precursor uptake was

Figure 11.

^3H -glucosamine label incorporated into hyaluronic acid in dorsal iris, ventral iris, and lens tissues of sham-lentectomized eyes at five intervals after surgery. Data points represent values from individual experiments based on homogenates of 50-60 pooled tissues. Where duplicate experiments were performed, the lines join mean values.



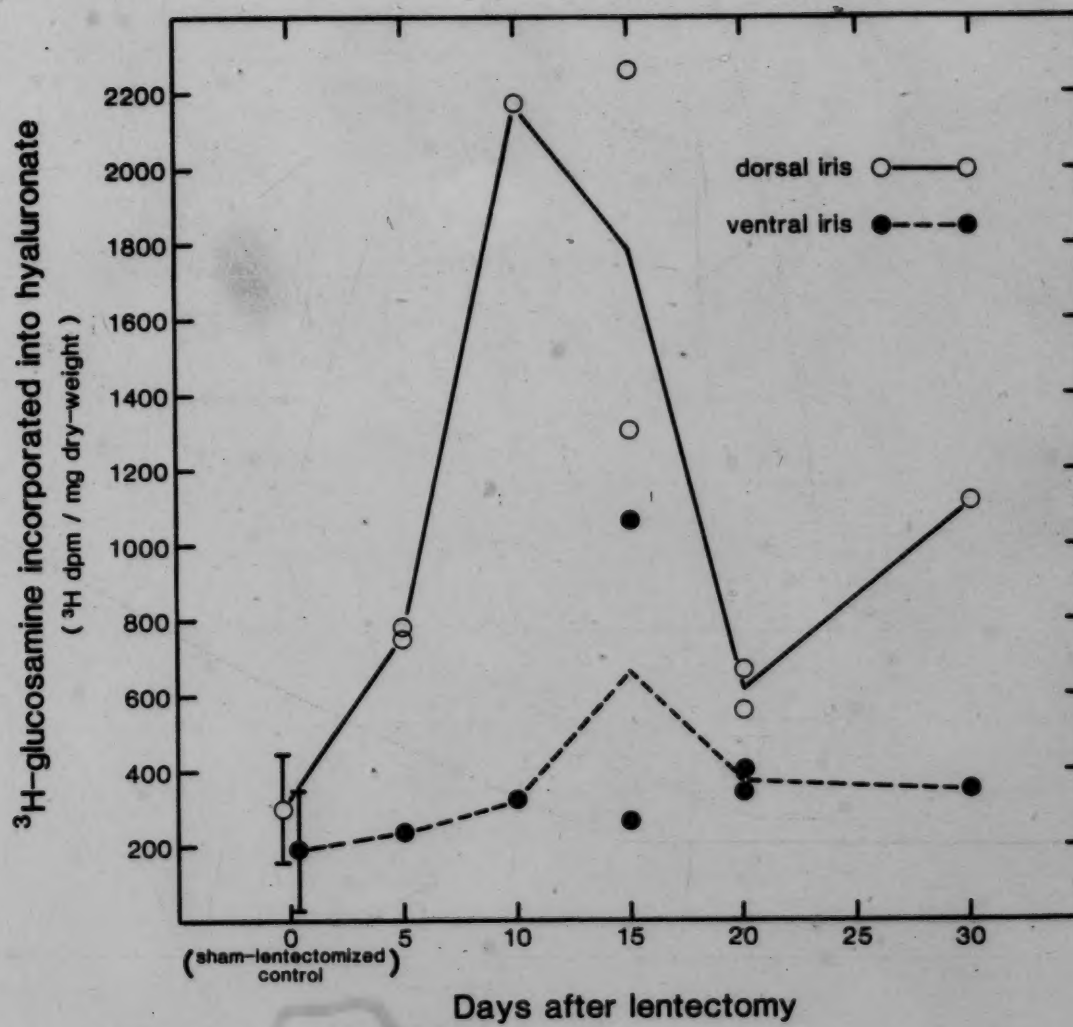
measured in one group of control ventral iris tissues at 15 days after sham-lentectomy. Owing to the small number of experiments performed, it is not clear whether these differences simply represent random variation about a common level of precursor uptake or whether labelling of hyaluronate within the control iris tissues is actually altered slightly as a function of time following the ocular trauma associated with the sham-lentectomy operation. Labelling of hyaluronate in the dorsal halves of the control iris tissues tended to be higher than in the ventral halves of irises from the sham-lentectomized eyes of the same animals, but this difference was not statistically significant ($0.05 \leq P \leq 0.10$; Wilcoxon's signed rank test). At all times after sham-lentectomy, the mature lenses of control eyes incorporated only negligible amounts of label into hyaluronate (Figure 11).

Within lentectomized eyes, labelling of hyaluronate was consistently higher in dorsal iris halves than in ventral iris halves from the same group of animals (Figure 12). Collectively the data demonstrated a significantly enhanced rate of precursor uptake into hyaluronate within the lens-forming (dorsal) region of the iris in comparison with the non-lens-forming (ventral) region during the process of lens regeneration ($P \leq 0.02$; Wilcoxon's signed rank test).

At all stages of lens regeneration, labelling of hyaluronate was consistently greater within the dorsal iris

Figure 12.

³H-glucosamine uptake into hyaluronate in dorsal iris and ventral iris halves at different stages of lens regeneration. Label uptake at 5, 10, 15, 20 and 30 days after lentectomy is compared to average label uptake in control dorsal and ventral iris halves of sham-lentectomized eyes. Data points from days 5 to 30 after lentectomy represent values from individual experiments based on homogenates of 50-60 pooled iris halves. Data points for control iris tissues represent the means $\pm$ 95% confidence limits for the pooled data from the contralateral sham-lentectomized eyes of all groups of experimental animals.



tissues of lentectomized eyes than in the control dorsal irises of the contralateral sham-lentectomized eyes from the same animals ($P \leq 0.01$; Wilcoxon's signed rank test). The rate of incorporation of ^3H -label into hyaluronate rose rapidly within the dorsal iris tissues during the first 10 days after lentectomy, and was maximal between days 10 and 15 of regeneration when precursor uptake was four to seven-fold higher than the average value for control dorsal irises (Figure 12). Labelling of hyaluronate declined markedly by 20 days after lentectomy, but recovered to a somewhat higher level by day 30.

Labelling of hyaluronate in the ventral irises of lentectomized eyes was consistently elevated above the level in the contralateral control ventral irises from the same animals ($P = 0.05$; Wilcoxon's signed rank test), but the magnitude of the difference was usually small (Figure 12). In most experiments the rates of precursor incorporation into hyaluronate were similar within the ventral iris tissues at different times after lentectomy, ranging from 1.4 to 2.2 times greater than the mean control level. However, an exceptionally high labelling of hyaluronate was measured in one group of ventral iris tissues from 15-day regenerate eyes.

B) *Labelling of Streptomyces*

Hyaluronidase-Resistant GAGs:

Although uptake of ^3H -glucosamine into hyaluronate was

demonstrable in all iris tissues, considerably greater amounts of the label were incorporated into GAGs that were resistant to digestion with *Streptomyces* hyaluronidase. These hyaluronidase-resistant GAGs would presumably consist of the various sulfated GAG types along with an undetermined proportion of unsulfated chondroitin polymers.

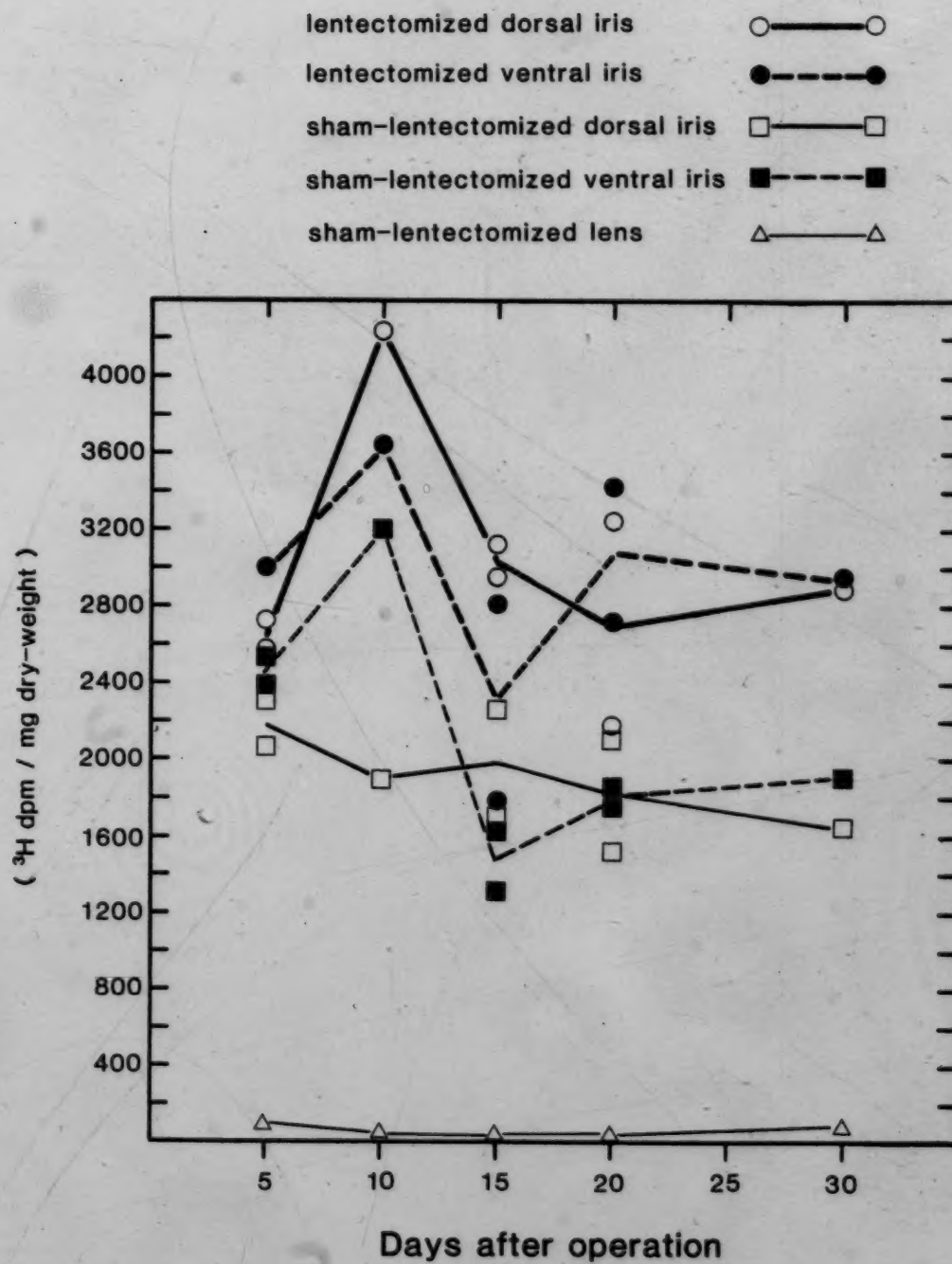
The amounts of label accumulated into hyaluronidase-resistant GAGs varied considerably at different times after ocular surgery in the irises of both lentectomized and sham-lentectomized eyes (Figure 13). Despite this variability, however, certain trends were apparent in the data. Uptake of ^3H -label into hyaluronidase-resistant GAGs was always greater in the dorsal iris tissues of lentectomized eyes than in the dorsal irises of contralateral sham-lentectomized eyes from the same animals. This difference was statistically significant ($P \leq 0.01$; Wilcoxon's signed rank test). Labelling of hyaluronidase-resistant GAGs was also consistently higher in the ventral irises of lentectomized eyes than in the ventral irises of sham-lentectomized eyes from the same animals (significant at $P \leq 0.02$).

There was no significant difference between the rates of labelling of hyaluronidase-resistant GAGs in the dorsal and ventral regions of irises from lentectomized eyes ($P \geq 0.10$; Wilcoxon's signed rank test). Similarly, labelling of these GAGs was not significantly different in the dorsal and ventral halves of irises from sham-lentectomized eyes ($P \geq 0.10$). Labelling of hyaluronidase-resistant GAGs was

Figure 13.

Uptake of ^3H label into *Streptomyces* hyaluronidase-resistant GAGs of dorsal iris, ventral iris, and lens tissues. Labelling is compared in the tissues of lentectomized and contralateral sham-lentectomized eyes at five intervals after surgery. Data points for iris halves represent values from single experiments based on 50-60 pooled tissues. Where duplicate experiments were performed, the lines join their mean values. Data points for the lens tissue of sham-lentectomized eyes represent either values of single experiments or mean values of duplicate experiments.

^3H label incorporated into
Streptomyces hyaluronidase-resistant GAGs



always very low in the mature lenses of sham-lentectomized control eyes (Figure 13).

These results indicate that labelled precursor uptake into newly synthesized hyaluronidase-resistant GAGs is augmented within the iris during lens regeneration in comparison with labelling of iris tissues subjected to ocular trauma without lens removal. The enhanced precursor incorporation is activated in both the lens-forming (dorsal) and non-lens-forming (ventral) regions of the iris.

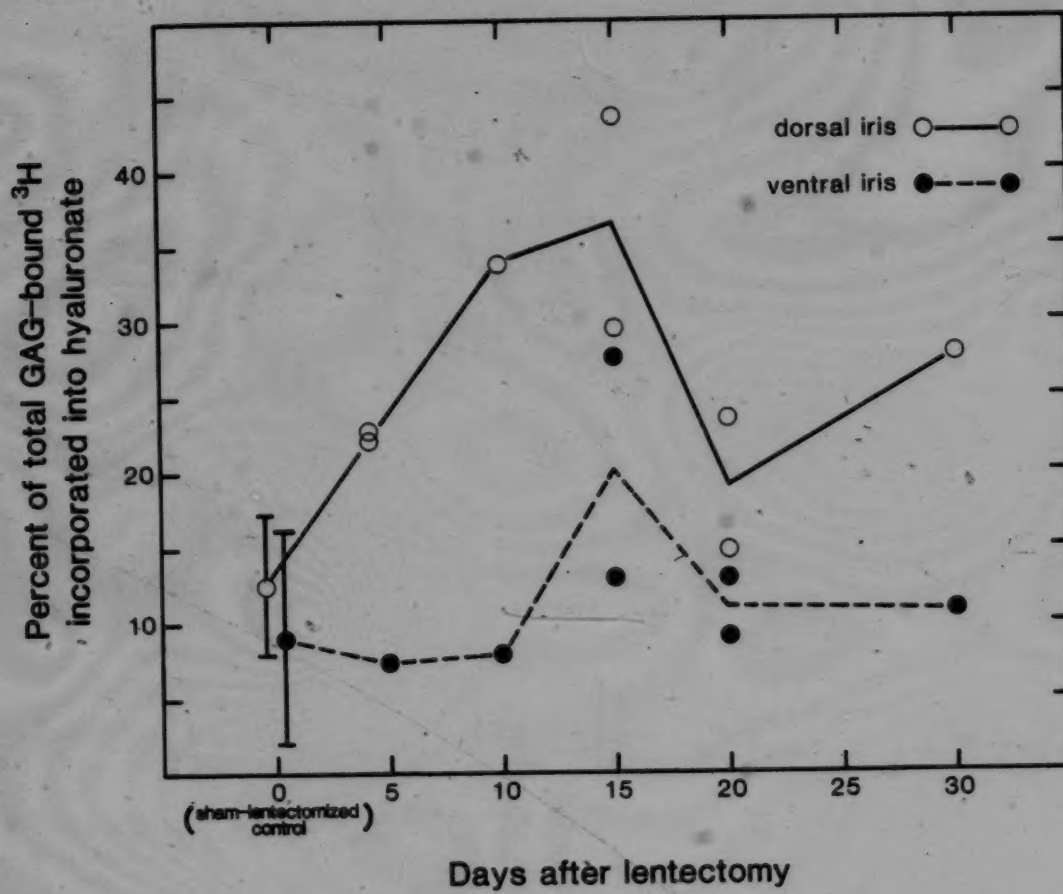
C) *Relative Proportions of Total ^3H -Glucosamine Label Incorporated into Hyaluronate at Different Stages of Lens Regeneration:*

Experimental data presented earlier (RESULTS--section I-B) indicated that, despite an overall elevation in the rate of $^{35}\text{SO}_4$ uptake into sulfated GAGs within the iris following lentectomy, the relative proportions of total label incorporated into different types of sulfated GAG remained virtually unchanged throughout the process of lens regeneration. In view of this relationship, it was desirable to estimate whether the proportion of total GAG-bound ^3H -glucosamine that was incorporated into hyaluronate was also held constant during lens regeneration or whether it varied in a stage-dependent manner.

In Figure 14, the amounts of ^3H -label incorporated into hyaluronate within dorsal and ventral irises at various times after lentectomy are plotted as percentages of

Figure 14.

Percentages of total GAG-bound ^3H -glucosamine label present in hyaluronate in dorsal and ventral iris halves at different stages of lens regeneration. Data points for iris tissue at days 5 to 30 after lentectomy represent values from single experiments based on 50-60 pooled tissues. Where duplicate experiments were performed, the lines join their mean values. Data points for control dorsal and ventral irises represent the means $\pm$ 95% confidence limits for data pooled from the contralateral sham-lentectomized eyes of all groups of experimental animals.



the total GAG-bound label in the tissues. Values for control dorsal and ventral iris tissues are the means ($\pm 95\%$ confidence limits) of pooled data from contralateral sham-lentectomized eyes.

Hyaluronate usually accounted for only a small proportion of total GAG-bound label in the control iris tissues of sham-lentectomized eyes. Within the dorsal and ventral regions of the control irises, the percentages of total label attributable to hyaluronate ($12.6 \pm 4\%$ and $9.1 \pm 6\%$, respectively) were not significantly different ($P \geq 0.10$; Wilcoxon's signed rank test).

Among lentectomized eyes, dorsal iris tissues consistently incorporated a greater proportion of GAG-bound label into hyaluronate than did the ventral iris tissues from the same animals (significant at $P \leq 0.02$). In the dorsal iris, the percentage of total label incorporated into hyaluronate rose above control levels during the first five days after lentectomy. At 10 day and 15 day regeneration stages, hyaluronic acid accounted for 30-43% of the total GAG-bound label in the dorsal iris. By 20 days after lentectomy the proportion of label attributable to hyaluronate declined markedly, although it recovered to a slightly higher value by day 30. Throughout lens regeneration, the dorsal iris tissues of lentectomized eyes consistently incorporated a greater proportion of total GAG-bound label into hyaluronate than did the contralateral control dorsal iris tissues of sham-lentectomized eyes from the same

animals (significant at $P \leq 0.01$; Wilcoxon's signed rank test).

In contrast, the proportions of total label associated with hyaluronate in ventral irises at regeneration stages of 5, 10, 20, and 30 days were similar to the average control value for non-regenerating ventral iris tissues. Only at 15 days after lentectomy was the percentage of hyaluronate-bound label elevated appreciably in the ventral iris tissues.

III. Distribution of $^{35}\text{SO}_4$ Label in Autoradiographs of the Newt Eye

Histological sections were prepared from normal and 80-day regenerate eyes of newts that had been labelled four days with $^{35}\text{SO}_4$ (METHODS--section IV). The tissue sections were processed for liquid emulsion autoradiography and then examined by light microscopy. Using this method, the predominant sites of ^{35}S label uptake were revealed by accumulations of silver grains in the overlying emulsion.

The distribution of ^{35}S label was qualitatively similar in normal and 80-day regenerate eyes. The silver grains were conspicuously concentrated above background levels in the vicinity of several ocular tissues. The highest grain densities were associated with the neural retina and lens in both normal and 80-day regenerate eyes, indicating that these were the major sites of ^{35}S deposition. Moderately high grain densities were associated with the iris and ciliary body, the cornea, and the sclera.

Within the iris, the silver grains were distributed over both the iris stroma and the inner and outer laminae of the pigmented iris epithelium (Figures 15, 16). No attempt was made to quantify the relative amounts of ^{35}S label present in the stromal and epithelial tissue compartments, as the dense melanin pigmentation in the iris epithelium prevented reliable counts of the overlying silver grains. The distribution and density of silver grains were

Figure 15.

(a) Autoradiograph of distribution of ^{35}S -sulfate label in the dorsal iris in a median sagittal section through a normal, non-lentectomized newt eye. Regions of the iris epithelium (ie), iris stroma (is), and lens (L) are indicated. Rectangles indicate the areas depicted in Figures 15b and 15c. Magnification is 420X.

(b) Autoradiograph of distribution of ^{35}S -sulfate label at the tip of the dorsal iris of a normal, non-lentectomized newt eye. Regions of iris epithelium (ie) and lens (L) are indicated. Magnification is 1530X.

(c) Autoradiograph of distribution of ^{35}S -sulfate label in a region of the dorsal iris distal to the pupillary margin. Regions of iris epithelium (ie), iris stroma (is), and lens (L) are indicated. Magnification is 1530X.

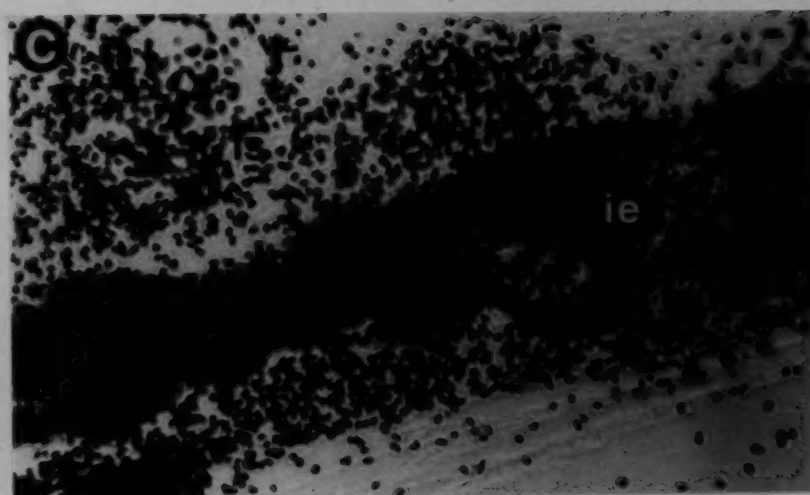
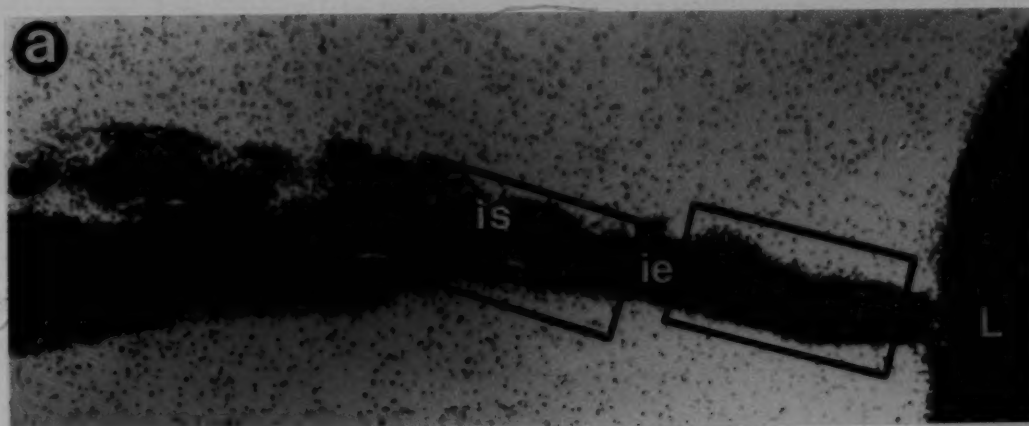


Figure 16.

(a) Autoradiograph of ^{35}S -sulfate label distribution in the dorsal iris in a median sagittal section of an eye at 80 days after lentectomy. Regions of the iris epithelium (ie), iris stroma (is), and regenerated lens (L) are indicated. Rectangles indicate the areas depicted in Figures 16b and 16c. Magnification is 420X.

(b) Autoradiograph of ^{35}S -sulfate distribution in a region of the dorsal iris adjacent to the lens regenerate. The iris epithelium (ie) and iris stroma (is) are indicated. Magnification is 1530X.

(c) Autoradiograph of ^{35}S -sulfate distribution in a region of the dorsal iris distal to the lens regenerate. The iris epithelium (ie) and iris stroma (is) are indicated. Magnification is 1530X.



not conspicuously different in the dorsal and ventral regions of the iris, although no quantitative comparisons were made.

IV. Degradation of ^3H -Chondroitin Sulfate Substrate (^3H -CSS) by Iris Tissue Extracts

As a preliminary approach to determining whether enzymes active in GAG catabolism were present in the iris during lens regeneration, cell-free extracts of pooled iris tissues were incubated with ^3H -labelled chondroitin sulfate substrate (^3H -CSS). The substrate was prepared from the whole bodies of newts that had been labelled *in vivo* with ^3H -glucosamine, using a series of standard fractionation procedures (METHODS--section V-B). As evidenced by its sensitivity to chondroitinase AC digestion, 69% of the label in the ^3H -CSS preparation was incorporated into chondroitin sulfate polymers of the chondroitins 4- and 6-sulfate types (Table 4). The major contaminant in the preparation was dermatan sulfate (resistant to chondroitinase AC but sensitive to chondroitinase ABC), which accounted for approximately 17% of the total label. There were lesser degrees of contamination by heparin/heparan sulfate (nitrous acid sensitive GAG) and by material that resisted both chondroitinase ABC and nitrous acid digestion (presumably keratan sulfate or glycoprotein).

Incubations of substrate with iris tissue extract were conducted at acid pH (pH 3.7) to approximate conditions that are optimal for chondroitin sulfate-degrading endoglycosidases from other vertebrate cell types (Meyer, 1971; Glaser and Conrad, 1979). The incubations were routinely

Table 4. Sensitivity of ^3H -CSS (^3H -labelled chondroitin sulfate substrate from newt tissue) to digestions with chondroitinase AC, chondroitinase ABC, and nitrous acid

Treatment	cpm recovered after mock digestion	cpm recovered after digestive treatment	Percent degradation
chondroitinase AC	787 721	246 222	68.7% 69.2%
chondroitinase ABC	787 699	114 91	85.6% 87.0%
nitrous acid	712	659	7.4%
chondroitinase ABC + nitrous acid	711	106	85.1%

performed for a prolonged period (24 hours) to maximize the possibility of detecting low rates of substrate hydrolysis. The extent of substrate degradation mediated by iris-derived enzyme activity was indicated by the amount of CPC-precipitable label recovered following incubation of ^3H -CSS with active iris tissue extract in comparison with the total label recovered following incubation of substrate with iris extract that had first been inactivated by boiling (METHODS--section V-C).

In each experiment performed, a lower proportion of CPC-precipitable label was recovered after incubation of ^3H -CSS with active iris extract than after the control incubation with heat-inactivated iris extract (Table 5). The results suggest that a portion (18-37%) of the original substrate was degraded to oligosaccharide fragments by a heat-labile enzymatic agent in the iris tissue. Owing to inadequacies in the experimental design and the small number of experiments conducted, it is not certain whether the differences in the percentages of substrate degraded by irises at different times after lentectomy were due to actual variation in chondroitin sulfate catabolic activity within the iris during lens regeneration.

Table 5. Degradation of ^3H -CSS by cell-free extracts of iris tissue homogenates

Regeneration stage	cpm recovered from control sample*	cpm recovered from test sample**	Percent ^3H -CSS degradation
Normal iris	3230	2384	26.2%
10 day	1471	1152	21.7%
15 day	1677	1058	36.9%
20 day	2280	1469	35.6%
20 day	2790	1806	35.3%
30 day	2738	2230	18.6%

*: in the control sample, ^3H -CSS was incubated for 24 h with heat-inactivated (boiled) iris extract.

**: in the test sample, ^3H -CSS was incubated for 24 h with active iris extract.

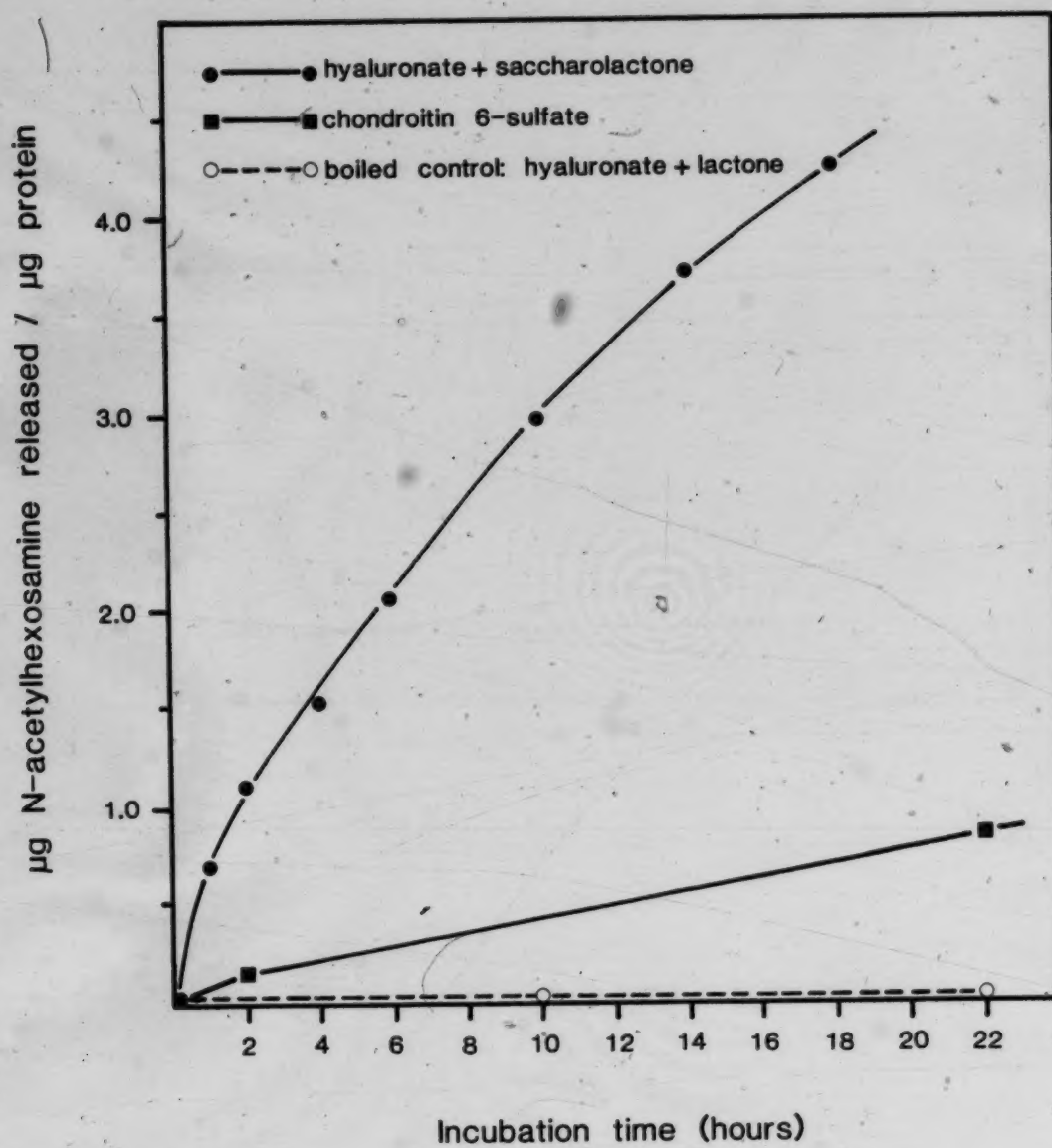
V. Endogenous Hyaluronidase Activity in the Newt Iris

In order to test for the presence of hyaluronidase activity in the newt iris, cell-free extracts of iris tissue homogenates were incubated with exogenous hyaluronic acid substrate (METHODS--section VI-B). The incubations were routinely conducted under conditions of acid pH (pH 3.7) and physiological salt concentration that have proven optimal for lysosomal hyaluronidases in a variety of vertebrate tissues (Bollet *et al.*, 1963; Aronson and Davidson, 1967b; Cashman *et al.*, 1969; Polansky *et al.*, 1974; Orkin and Toole, 1980; Sampaio and Dietrich, 1981). To prevent digestion of the hyaluronate substrate by the sequential attack of β -glucuronidase and β -N-acetylhexosaminidase exoglycosidases, the incubations were usually performed in the presence of D-saccharic acid 1,4-lactone (saccharolactone), a potent and specific inhibitor of β -glucuronidase action (Levy and Marsh, 1959). Hyaluronate degradation in the iris extract reaction mixtures was monitored by colorimetric determination of the N-acetylhexosamine end-groups liberated during cleavage of the internal N-acetylglucosaminidic bonds in the substrate polymers (METHODS--section VI-C).

In the presence of saccharolactone, cell-free extracts of iris tissue from normal, non-lentectomized newts exhibited a high level of degradative action upon exogenous hyaluronate substrate (Figure 17). Release of N-acetylhex-

Figure 17.

Time course of digestion of hyaluronate and chondroitin 6-sulfate substrates by the cell-free extracts of a normal iris tissue homogenate. Extent of GAG polymer degradation is expressed as micrograms of reducing N-acetylhexosamine end-groups released per microgram of iris extract protein. Incubations were at room temperature in 0.1 M sodium formate/0.15 M NaCl (pH 3.7). Hyaluronate substrate was incubated in the presence of saccharic acid 1,4-lactone (saccharolactone=lactone). Saccharolactone was absent from incubations with chondroitin sulfate substrate. Controls involved incubation of hyaluronate substrate with iris extract that had been heat-inactivated by boiling.



osamine end-groups from hyaluronate was particularly rapid during the first two hours of incubation at room temperature and pH 3.7. With continued incubation over an 18-hour period, there was a moderate but progressive decline in the rate of formation of additional N-acetylhexosamine end-groups. Release of N-acetylhexosamine from an equivalent amount of purified chondroitin 6-sulfate substrate proceeded at only 15-20% of the rate of degradation of hyaluronate substrate (Figure 17). There was no release of N-acetylhexosamine in control experiments in which hyaluronate substrate was incubated for up to 22 hours with iris extract that had first been heat-inactivated by boiling. These results indicated the presence of a hyaluronidase of the endo- β -hexosaminidase type within normal iris tissue extracts.

To ascertain whether the level of hyaluronidase activity was altered during lens regeneration, the extents of hyaluronate substrate degradation after two-hour and six-hour incubation intervals were compared in extracts of normal irises and irises at 10, 15, and 30 days after lenticectomy (Table 6). The data indicate that there were no marked differences in the amounts of N-acetylhexosamine released at different stages of lens regeneration under the same incubation conditions. Accordingly, hyaluronidase activity in the iris tissue extracts appears to remain reasonably stable throughout the regeneration process.

Following a two-hour incubation period, the extent of

Table 6. Hyaluronidase activities of cell-free extracts of iris tissues at different stages of lens regeneration

Regeneration Stage	Incubation Time	µg GlcNac released per iris from hyaluronate substrate	
		saccharic acid 1,4-lactone present	saccharic acid 1,4-lactone absent
Normal iris	2 h	1.00±0.04*	1.09
	6 h	1.98±0.06	N.D.
	6 h (boiled control)	0.03±0.03*	N.D.
10 day	2 h	1.08	1.10
	6 h	2.30	N.D.
	6 h (boiled control)	0.07	N.D.
15 day	2 h	1.20	1.33
	6 h	1.99	N.D.
	6 h (boiled control)	0.10	N.D.
30 day	2 h	1.22	1.46
	6 h	2.57	N.D.
	6 h (boiled control)	0.10	N.D.

GlcNac: N-acetylglucosamine

*: value represents mean ± standard error of duplicate determinations

N.D.: indicates determinations were not performed

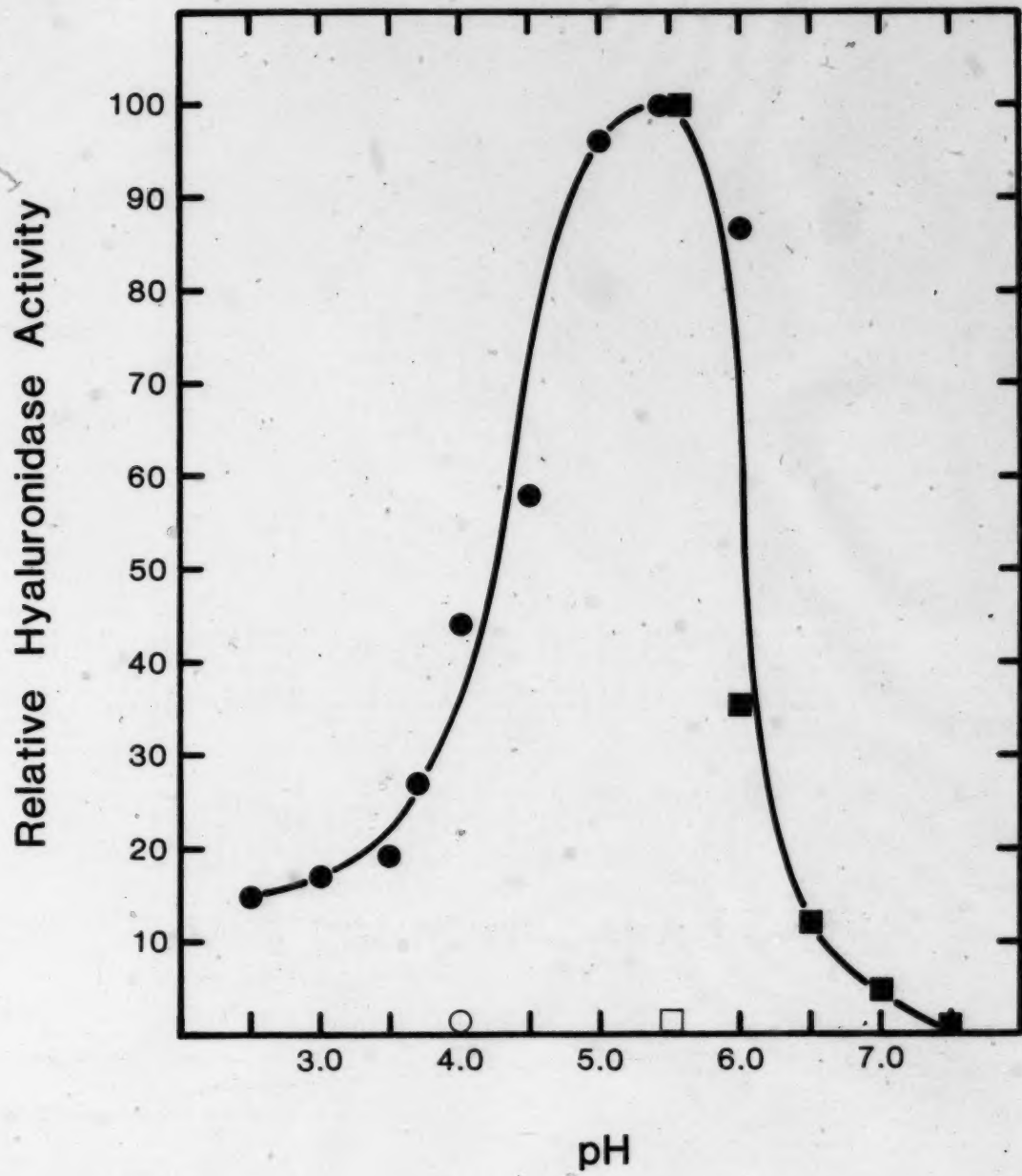
hyaluronate substrate degradation by iris tissue extract was only marginally elevated when saccharolactone was absent from the reaction mixtures in comparison to when the β -glucuronidase inhibitor was present (Table 6). This implies that digestion of hyaluronate or hyaluronate-oligosaccharides by step-wise exoglycosidase action was negligible at the pH and ionic conditions of the incubation mixture.

The pH optimum of the iris extract hyaluronidase was determined by comparing the release of N-acetylhexosamine end-groups from hyaluronate substrate under a series of different pH conditions (METHODS--section VI-D). The enzyme exhibited a narrow pH optimum at pH 5.0-5.5 (Figure 18). There was little activity below pH 3.5 and negligible activity above pH 6.5.

As a control to determine whether the hydrolysis of exogenous hyaluronate in iris tissue extracts was attributable to hyaluronidases produced by contaminating microorganisms, normal iris tissue extract was incubated 18 hours with hyaluronate substrate. The resulting digest was examined spectrophotometrically for the presence of an ultraviolet absorption peak at 235 nm, as is characteristic of the Δ -4,5-unsaturated uronide degradation products of microbial hyaluronidase action (METHODS--section VI-E). Despite the formation of a high concentration of N-acetylhexosamine end-groups, the absorbance of the digest at 235 nm was no greater than in a control mixture in which heat-

Figure 18.

Comparison of the hyaluronate-degrading activities of iris tissue extracts at different pH values. Incubations were at room temperature with hyaluronate substrate. The relative hyaluronidase activity represents micrograms of N-acetylhexosamine end-groups released expressed as a percentage of the maximum product formation in each experimental series. Circles represent incubations performed in 0.1 M formate/0.15 M NaCl buffers. Squares represent incubations performed in 0.1 M phosphate/0.15 M NaCl buffers. Open symbols represent values of control experiments using iris extract that had been heat-inactivated by boiling. Closed symbols represent values from experiments using active iris extracts.



inactivated iris extract was incubated with hyaluronate substrate (Table 7). Wavelength scanning of the iris extract reaction mixture (using the boiled extract control mixture as a blank) demonstrated the complete absence of an absorption peak at 235 nm (Figure 19a). In contrast, following incubation of hyaluronate substrate with *Streptomyces* hyaluronidase (an endohexosaminidase of microbial origin), there was strong absorbance at 235 nm that was not observed on incubation of the substrate with heat-inactivated *Streptomyces* enzyme (Table 7). Scanning of the *Streptomyces* hyaluronidase incubation mixture (using the boiled enzyme control mixture as a blank) confirmed the presence of a prominent absorption peak at 235 nm (Figure 19b), indicative of Δ -4,5-unsaturated uronide formation. These results demonstrate that the hyaluronidase activity associated with iris extracts was due to an enzyme endogenous to the newt iris tissue and was not attributable to microbial action.

Table 7. Absorbance at 235 nm of digestion products formed after 18 h incubation of hyaluronate substrate with iris tissue extract or *Streptomyces* hyaluronidase

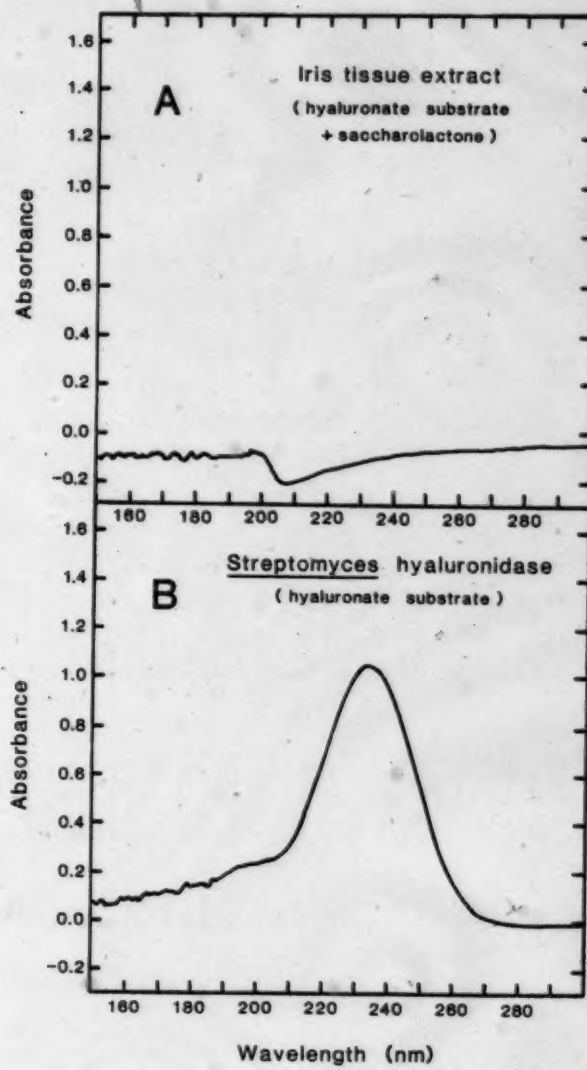
Treatment	Absorbance at 235 nm	μ g. GlcNac released
iris extract (boiled control)	0.550	N.D.
iris extract (test sample)	0.447	24.6
<i>Streptomyces</i> hyaluronidase (boiled control)	0.028	N.D.
<i>Streptomyces</i> hyaluronidase (test sample)	1.080	N.D.

N.D.: indicates that N-acetylglucosamine (GlcNac) end-group determination was not performed

Figure 19.

(a) Absorbance spectrum of digestion products of hyaluronate substrate incubated 18 hours with iris tissue extract. Incubation was at pH 3.7 and room temperature in the presence of saccharic acid 1,4-lactone.

(b) Absorbance spectrum of digestion products of hyaluronate substrate incubated 18 hours with *Streptomyces* hyaluronidase. Incubation was at pH 5.0 and 60°C.



DISCUSSION

1. Interpretation of Results

A) *General Considerations:*

The foregoing studies have focussed on examining the patterns of radioactive precursor uptake into the various molecular species of glycosaminoglycans in the newt iris during the process of lens regeneration. The goal of these investigations was to determine whether changes in the types or amounts of GAGs produced within the iris tissue were temporally correlated with the dedifferentiation of iris epithelial cells, their conversion into lens fibers, or their redifferentiation into pigmented melanocytes.

The analytical approach of measuring rates of radioactive precursor uptake as indices of GAG synthetic activity was adopted in preference to less sensitive methods of direct chemical analysis of the GAG constituents of the newt iris because of the small quantities of tissue available for study (less than 100 μg lipid-free dry weight per iris). Measurements by Knepper *et al.* (1981) indicate that the rabbit iris, for example, contains only 3.5 μg of total GAG per mg of defatted tissue dry weight.

The additional sensitivity afforded by precursor labelling studies is not acquired, however, without certain limitations to the accuracy with which the experimental

data can be interpreted in terms of macromolecular synthesis *in situ*. Manasek (1975) has discussed a number of the problems that accompany the use of radioactively labelled precursors for the analysis of GAG composition and synthesis in developmental systems. These problems include the potential for metabolic interconversion of labelled monosaccharide precursors, the possibility of variation in the specific activities of intracellular precursor pools in different tissues and at different stages of development, and the uncertainty that equimolar amounts of a labelled precursor will be incorporated into different types of GAG disaccharide subunits. In critically interpreting the results from the present studies, due consideration will be given to the various limitations which restrict the inferences that can be accurately drawn from the experimental data. Nevertheless, in a number of instances the interpretations will of necessity entail the acceptance of certain reasonable assumptions regarding the mechanisms of GAG synthesis in the newt iris based on the general patterns established in other tissue systems.

B). *Evidence of Sulfated GAG Synthesis in the Normal Iris:*

The fact that radioactive sulfate label is incorporated into ethanol-insoluble, CPC-precipitable material in the irises of both unoperated and lentectomized eyes (RESULTS--section I-A) indicates that the newt iris tissue synthesizes sulfated glycosaminoglycans during its normal

metabolism as well as during lens regeneration. The comparatively low level of $^{35}\text{SO}_4$ uptake into GAGs in the normal iris suggests that the iris tissue of the untraumatized eye has a continuous basal rate of new GAG polymer synthesis. It is reasonable to assume that the content of GAGs in the mature iris is normally maintained in a steady state by an equivalent rate of GAG catabolism, such that there is a continuous turnover of the polysaccharides in the tissue. If the rate of GAG production in the iris were to exceed its rate of degradation for any appreciable length of time, the GAGs would progressively accumulate and eventually disrupt the architecture and function of the tissue. This situation is exemplified by the "mucopolysaccharidoses" or mucopolysaccharide storage diseases in humans in which hereditary deficiencies in the enzymes participating in GAG catabolism cause an intracellular accumulation of incompletely degraded GAG polymers and result in a variety of anatomical and physiological anomalies including skeletal deformities, mental retardation, and defects of the heart, liver, and spleen (Dorfman and Matalon, 1972).

A normal basal turnover of GAGs, as proposed for the untraumatized newt iris, is a general feature of GAG and proteoglycan metabolism in a variety of vertebrate tissues (Gallagher, 1977). The half-lives of the GAG polymers *in situ* vary widely with the animal and tissue source, but are generally long (8-80 days) for the sulfated GAGs of mammalian connective tissues such as cartilage and dermis

(Bostrom, 1952; Schiller *et al.*, 1956; Handley and Phelps, 1972; Gross *et al.*, 1960; Lohmander *et al.*, 1973) and may be shorter (2-10 days) for the GAGs of other tissues such as brain and kidney (Margolis and Margolis, 1973; Barry and Bowness, 1975). In cultured dermal and arterial fibroblasts, the half-lives of GAGs in intracellular pools can be as short as 4-8 hours (Fratantoni *et al.*, 1968; Kresse *et al.*, 1975a). The principal sites of the GAG degradation in vertebrate tissues are the lysosomes, which contain a battery of enzymes for digestion of the various GAG polymers to monosaccharide subunits (Barrett and Heath, 1977).

If, as suggested, the content of GAGs in the normal iris is maintained through a balance between a basal level of polysaccharide assembly and an equivalent level of polymer degradation, it follows that a perturbation of this balance (*e.g.* an increase in either the rate of GAG synthesis or its rate of catabolism) would initiate a pattern of either net accumulation or net loss of GAG in the iris and might serve as a mechanism of modulating tissue structure or function.

C) *Evidence of Enhanced Synthesis of Sulfated GAGs
in the Iris during Lens Regeneration:*

The dramatic elevation in the rate of $^{35}\text{SO}_4$ uptake into CPC-precipitable material in the iris following lens removal (RESULTS--section I-A) suggests that the morphological changes in the iris during lens regeneration are

temporally correlated with a period of enhanced sulfated GAG production in the tissue. This net increase in sulfated GAG synthesis could arise from either an elevated rate of new GAG polymer assembly or a reduced rate of labelled polymer degradation (*i.e.* a prolongation of the half-life of newly synthesized polymers).

Differences in the average rate of $^{35}\text{SO}_4$ uptake at different times after lentectomy (RESULTS--section I-A) suggest a stage-dependent variation in the rate of sulfated GAG production in the iris during the process of lens regeneration. Enhanced sulfated GAG synthesis was evident by the fifth day after lens removal and persisted until at least day 30 of regeneration. The level of net sulfated GAG synthesis appeared maximal at approximately 15 days after lentectomy.

It is not likely that the elevated $^{35}\text{SO}_4$ uptake in the iris during lens regeneration was simply the result of an increase in the specific activity of the intracellular sulfate precursor pool. The uptake of ^3H -glucosamine into GAGs that resisted *Streptomyces* hyaluronidase digestion (predominantly sulfated GAGs) was also increased following lentectomy (RESULTS--section II-B) despite the fact that the glucosamine and sulfate precursors are sequestered in metabolically independent pools (Manasek, 1975). Direct measurements of the sizes of the endogenous sulfate or glucosamine pools would be required to conclusively discount the possibility of altered precursor pool activities, but

these were not considered feasible due to the prohibitively small amounts of tissue available.

The possibility must also be considered that the increased $^{35}\text{SO}_4$ uptake in the iris following lentectomy might reflect an increase in the degree of sulfation of GAG polymers, rather than an actual increase in the amount of polymer synthesis. However the increase in ^3H -glucosamine incorporation into *Streptomyces* hyaluronidase-resistant GAGs following lentectomy (RESULTS--section II-B) indicates that there was indeed an augmentation of the quantity of polysaccharide chain production, since the ^3H -glucosamine label is limited to one hexosamine residue per repeating disaccharide subunit. Nevertheless, it is interesting that whereas the rate of $^{35}\text{SO}_4$ uptake into sulfated GAGs was elevated two to three-fold in irises of lentectomized eyes when compared with unoperated eyes, the uptake of ^3H -glucosamine into *Streptomyces* hyaluronidase-resistant GAGs was increased by an average factor of only 1.6 times in irises of lentectomized eyes as compared with sham-lentectomized controls. This discrepancy might be indicative of a regeneration-related increase in the average sulfate content of one or more of the sulfated GAG species of the iris that accompanies the overall increase in the rate of GAG polymer synthesis. Alternatively, the data may indicate that the average rate of sulfated GAG synthesis in the traumatized irises of sham-lentectomized eyes (used as controls for the ^3H -glucosamine uptake studies) was somewhat higher than the

rate of sulfated GAG synthesis in the untraumatized irises of unoperated eyes (used as $^{35}\text{SO}_4$ uptake controls). Confirmation of the first hypothesis would require comparison of the sulfated GAGs of regenerating and normal iris tissues by a method that distinguishes differences in molecular charge density (e.g. electrophoresis on cellulose acetate membranes or ion exchange chromatography). Confirmation of the second hypothesis would require comparison of the rates of precursor uptake between irises of sham-lentectomized eyes and irises of contralateral unoperated eyes from the same animals.

D) *Evidence of Chondroitin Sulfate, Dermatan Sulfate, and Heparin/Heparan Sulfate Production in Normal and Regenerating Iris Tissue:*

On the basis of their differing sensitivities to chondroitinase AC, chondroitinase ABC, and nitrous acid treatments, the $^{35}\text{SO}_4$ -labelled GAGs of the newt iris could be divided into three broad classes:

[1] Chondroitin sulfate (C4S/C6S) type:

In the normal iris and at each stage of regeneration, 16-29% of the total GAG-bound $^{35}\text{SO}_4$ label was sensitive to chondroitinase AC digestion and resistant to nitrous acid hydrolysis (RESULTS--section I-B). For the sake of simplicity, this $^{35}\text{SO}_4$ -labelled fraction was designated as chondroitin sulfate. Since chondroitinase AC can hydrolyze any

internal $\beta 1 \rightarrow 4$ linkages between adjacent N-acetylhexosamine and D-glucuronate residues in GAG polymers, a number of structures could theoretically serve as substrates for the enzyme (Yamagata *et al.*, 1968, Taniguchi, 1976). Accordingly, any one or combination of the following structures could represent the material classified as chondroitin sulfate in the present study:

- homopolymers of chondroitin 4-sulfate disaccharides (*i.e.* authentic chondroitin 4-sulfate)
- homopolymers of chondroitin 6-sulfate disaccharides (*i.e.* authentic chondroitin 6-sulfate)
- chondroitin sulfate heteropolymers containing both galactosamine 4-sulfate and galactosamine 6-sulfate (*i.e.* hybrid chains of chondroitin 4-sulfate and chondroitin 6-sulfate disaccharides)
- chondroitin sulfate polymers having any of the above structures and displaying oversulfation or undersulfation of galactosamine residues or sulfation of D-glucuronate residues
- regions of copolymeric chondroitin sulfate/dermatan sulfate polymers containing closely spaced D-glucuronate residues (*i.e.* D-glucuronate-rich chondroitin sulfate/dermatan sulfate hybrids of the type described by Coster and Fransson, 1981).

[2] Dermatan sulfate (DS) type:

From 11-22% of total GAG-bound $^{35}\text{SO}_4$ label in the iris

tissue was associated with material that resisted chondroitinase AC treatment but was digested with chondroitinase ABC (RESULTS--section I-B). The latter enzyme is able to hydrolyze the $\beta 1 \rightarrow 4$ glycosidic bonds between galactosamine 4-sulfate and L-iduronate moieties as found in dermatan sulfate (Yamagata *et al.*, 1968). Material in this fraction could represent homopolymers of dermatan sulfate disaccharides, regions of hybrid dermatan sulfate/chondroitin sulfate polymers that lack D-glucuronate groups, and any over- or under-sulfated variants of the above.

[3] Heparin/heparan sulfate (Hep/HS) type:

The majority (approximately 60%) of GAG-bound $^{35}\text{SO}_4$ label in both normal and regenerating irises was resistant to chondroitinase AC digestion and sensitive to nitrous acid hydrolysis by the method of Lagunoff and Warren (1962) (RESULTS--section I-B). Under the conditions employed in this study, the nitrous acid reaction specifically cleaves the glycosidic linkages of N-sulfated or N-unsubstituted amino sugars (Lagunoff and Warren, 1962; Shively and Conrad, 1976). Since N-unsubstituted hexosamines are not found in glycosaminoglycans and N-sulfated hexosamines are limited to GAGs of the heparin and heparan sulfate types (INTRODUCTION--section III-A), the nitrous acid sensitive material is classified as heparin/heparan sulfate. No attempt was made to determine whether the structure of the iris tissue GAG corresponded more closely to that of heparan sulfate or of authentic heparin.

The proportions of total $^{35}\text{SO}_4$ label attributable to each of the chondroitin sulfate, dermatan sulfate, and heparin/heparan sulfate classes of GAG were remarkably similar both prior to lentectomy and at all stages of regeneration (RESULTS--section I-B). Apparently, the augmentation of GAG production in the iris that followed lentectomy did not alter the relative rates of synthesis of the different sulfated GAG species. The relative amounts of $^{35}\text{SO}_4$ uptake by each variety of GAG (*i.e.* approximately 60% for Hep/HS and 15-25% for each of C4S/C6S and DS) might be indicative of their actual relative rates of production in the iris, since all these polymers derive their sulfate groups from the same intracellular pool of 3'-phosphoadenosine 5'-phosphosulfate precursors (De Meio, 1967). It is by no means certain, however, that the different types of sulfated GAGs within the iris incorporate equimolar amounts of $^{35}\text{SO}_4$ label per repeating disaccharide subunit. Although chondroitin sulfates from many tissues conform to a formula of one sulfate group per disaccharide subunit, there are numerous examples of both oversulfated chondroitin sulfate variants (Suzuki, 1960; Mathews, 1975; Seno *et al.*, 1974; Kawai *et al.*, 1966; Suzuki *et al.*, 1968) and undersulfated forms (Kvist and Finnegan, 1970; Juvani *et al.*, 1975; Liao *et al.*, 1978). Similarly, heparin and heparan sulfate polymers from different sources exhibit considerable heterogeneity with respect to sulfate content (INTRODUCTION--section III-A) ranging from an average of less than one

sulfate group per disaccharide to an average of two or more sulfate groups per disaccharide subunit (Lamberg and Stool-miller, 1974). Accordingly, in the absence of information regarding the comparative sulfate contents of the different GAG species in the newt iris, one cannot be certain that ratios of $^{35}\text{SO}_4$ uptake provide a quantitative measure of the relative rates of chondroitin sulfate, dermatan sulfate and heparin/heparan sulfate synthesis in the tissue. Nevertheless, the data indicate that some stable relationship is maintained between the rates of production of the different sulfated GAG types within the iris after lens removal. This suggests that there is some physiological mechanism by which the rates of synthesis of the various sulfated GAG species are jointly coordinated in the iris tissue. It is possible that the chondroitin sulfate, dermatan sulfate, and heparin/heparan sulfate polymers are assembled *in situ* as subunits of a large proteoglycan macromolecule possessing a fixed complement of polysaccharide chains. The production of this proteoglycan complex might be amplified during lens regeneration, thereby increasing the synthetic rate of each of its constituent GAG moieties while preserving a constant relationship between their rates of synthesis. Proteoglycans with a high content of heparan sulfate have been isolated from liver plasma membranes (Oldberg *et al.*, 1979) as well as from the basement membranes of mammary epithelium (David and Bernfield, 1982), the renal glomeruli (Kanwar *et al.*, 1981), and a murine sarcoma

(Hassell *et al.*, 1980). Furthermore, the rate of sulfated GAG synthesis in tissues is often regulated by the availability of proteoglycan core proteins with substituent xylose residues that serve as initiation sites for GAG chain assembly (Roden, 1980). This is demonstrated by the increase in GAG polymer production which frequently follows addition of exogenous free β -D-xyloside groups to cells *in vitro* (Schwartz, 1977; Johnston and Keller, 1979). It must be acknowledged, however, that the presence of both chondroitin sulfate and heparan sulfate chains on a common core protein has not yet been demonstrated in any tissue (see DISCUSSION--section II, p 218).

E) *Evidence of Hyaluronate Production in the Iris:*

Enhanced Synthesis during Dedifferentiation:

The production of hyaluronate by newt iris tissue was demonstrated by ^3H -glucosamine uptake into ethanol insoluble, CPC-precipitable GAG that was sensitive to *Streptomyces* hyaluronidase digestion (RESULTS--section II-A). *Streptomyces* hyaluronidase is known to specifically degrade hyaluronate polymers while leaving GAGs of all other types intact (Ohya and Kaneko, 1970).

Incorporation of ^3H -glucosamine into hyaluronate was low in irises of sham-lentectomized eyes (RESULTS--section II-A), suggesting that the non-regenerating iris tissue has a low basal rate of hyaluronate synthesis. Hyaluronic acid accounted for an average of only 11% of total ^3H -glucos-

amine uptake into GAGs in sham-lentectomized eyes (RESULTS--section III-C), suggesting that the level of hyaluronate production in the non-regenerating iris was much lower than the combined rate of synthesis of other GAGs (*i.e.* *Streptomyces* hyaluronidase resistant types). The latter would presumably consist of the various sulfated GAG species already demonstrated in the newt iris (heparin/heparan sulfate, chondroitin sulfates, and dermatan sulfate) together with a possible undetermined component of unsulfated chondroitin polymers. Accordingly, hyaluronic acid may be a quantitatively minor synthetic component of the non-regenerating iris in comparison with sulfated GAG production.

The increase in hyaluronate labelling that followed lentectomy (RESULTS--section II-A) suggests that the net rate of hyaluronic acid synthesis in the iris is enhanced during lens regeneration. The consistently higher labelling of hyaluronate in dorsal as compared with ventral iris halves suggests that there is a topographical control over the rates of hyaluronic acid production in the lens-forming and non-lens-forming regions of the tissue. The rate of ^3H -glucosamine uptake into newly synthesized hyaluronate polymers was maximal in the dorsal iris at 10 to 15 days after lentectomy, corresponding to the period of most pronounced dedifferentiation in the iris epithelial cell population (INTRODUCTION--section II-D). During this period hyaluronate accounted for an average of 35% of total GAG-

bound ^3H -glucosamine label in the dorsal iris (RESULTS--section II-C), suggesting that hyaluronic acid may be one of the quantitatively major GAG species produced in the dedifferentiating dorsal iris. Ventral iris tissues demonstrated a comparatively small increase in hyaluronic acid labelling over the same time period (RESULTS--section II-A). Cumulatively, the results favour the interpretation that there is a pronounced increase in the ratio of hyaluronate to sulfated GAG synthesis in the dorsal (lens-forming) region of the iris following lentectomy. This ratio attains its maximum at the time of iris epithelial cell dedifferentiation. It is conceivable that an alteration in the relative proportions of hyaluronate and sulfated GAG accumulating in the iris could significantly modify the physico-chemical properties of the intercellular matrices.

As with the sulfated GAGs discussed previously, the increase in the net rate of hyaluronic acid synthesis in the iris tissue could be regulated at the cellular level through either an augmented rate of polysaccharide assembly or a reduced rate of polymer degradation. Unlike the sulfated GAG types, hyaluronic acid is not usually found in covalent association with protein in its native form (INTRODUCTION--section III-A), although hyaluronate-protein complexes may be transient biosynthetic precursors of hyaluronate in some cell types (Mikuni-Takagaki and Toole, 1981). Accordingly, it is likely that hyaluronate polymers

accumulate in the iris tissue matrices as free polysaccharide chains.

F) *Evidence of Endogenous Hyaluronidase*

Activity in the Newt Iris:

Among the most important agents of GAG breakdown in vertebrate tissues are the "hyaluronidases". These enzymes attack the internal $\beta 1 \rightarrow 4$ N-acetylhexosaminidic linkages within polymers of hyaluronic acid, chondroitin, chondroitin sulfate and, to a lesser extent, dermatan sulfate (Meyer, 1971). First demonstrated in the mammalian testis (Duran-Reynals, 1929; Chain and Duthie, 1940), hyaluronidase activity has been more recently identified in a wide variety of animal tissues and body fluids (e.g. Cobbin and Dicker, 1962; Bollet *et al.*, 1963; Aronson and Davidson, 1967b; Vaes, 1967; Toole, 1973).

Data from the present studies clearly demonstrate the presence of hyaluronidase activity in the normal and regeneration-activated newt iris (RESULTS--sections IV, V). Preliminary experiments suggested that a crude ^3H -labelled chondroitin sulfate substrate prepared from newt tissue was partially degraded to CPC-soluble fragments after prolonged incubation with extracts of homogenized iris tissue (RESULTS--section IV). Subsequent experiments employed the colorimetric assay of Reissig *et al.* (1955) to quantify the extent of GAG polymer digestion by iris tissue extracts and to characterize the nature of the polysaccharidase activity

in the newt iris (RESULTS--section V). The method measures the reducing N-acetylhexosamine end groups formed during cleavage of the hexosaminidic linkages in polymeric hyaluronate or chondroitin 6-sulfate substrates (Barrett and Heath, 1977). Incubation of 300 μ g of exogenous hyaluronate with cell-free iris extract resulted in rapid release of acetylhexosamine end-groups, demonstrating extensive hydrolysis of the substrate polymers. Hyaluronate digestion proceeded progressively from 60 min to 18 h of incubation at room temperature and pH 3.7. The enzymatic nature of the reaction is indicated by the fact that a brief boiling of the iris extract completely abolished its ability to subsequently degrade hyaluronate substrate.

The ability of iris extracts to degrade chondroitin sulfate in addition to hyaluronate was confirmed using exogenous chondroitin 6-sulfate substrate. The much lower rate of acetylhexosamine release from chondroitin 6-sulfate in comparison with hyaluronate is consistent with the behaviour of mammalian testicular hyaluronidases which typically degrade chondroitin sulfate at 1/6 to 1/15 the rate of hyaluronate (Meyer, 1971).

The enzymatic hydrolysis of hyaluronate and chondroitin sulfate is not in itself conclusive evidence of hyaluronidase activity, since GAG breakdown could also result from exoglycosidases of the β -N-acetylhexosaminidase and β -glucuronidase types that sequentially remove alternating hexosamine and glucuronate residues from the non-reducing

ends of the substrate polymers (Thompson *et al.*, 1973; Weissmann *et al.*, 1975). Indeed, Idoyaga-Vargas and Yamada (1974) have demonstrated the presence of β -N-acetylhexosaminidase activity in normal and regenerating newt iris tissue. Nevertheless, it is not likely that exoglycosidases contribute substantially to the hyaluronate-degrading activity demonstrated in the present study. All incubation buffers contained 0.15 M NaCl, which has been shown to strongly inhibit the actions of β -hexosaminidase and β -glucuronidase on polymeric hyaluronate substrates (Cashman *et al.*, 1969; Weissmann *et al.*, 1975). Moreover, incubations were routinely performed in the presence of saccharic acid 1,4-lactone, a powerful and specific inhibitor of β -glucuronidase (Levy and Conchie, 1966). The concentration of lactone inhibitor used in the present study (2.5 mM) was at least 100 times in excess of that required to inhibit mammalian β -glucuronidase (Levy and Marsh, 1959). It can be concluded that the hyaluronate-degrading activity of the iris tissue extracts is due to endoglycosidase rather than exoglycosidase action.

The formation of coloured reaction product in the assay of Reissig *et al.* (1955) confirms that the sites of the iris endoglycosidase attack are hexosaminidic linkages. Accordingly, the enzyme can be classified as a hyaluronidase of the β 1 $\rightarrow$ 4 endo-N-acetylhexosaminidase type. All known animal tissue hyaluronidases are endohexosaminidases (Meyer, 1971) with the exception of a hyaluronate-degrading

endoglucuronidase from leech salivary glands (Linker *et al.*, 1957).

The possibility that the hyaluronidase activity of the iris tissue extracts might have resulted from bacterial contamination can be readily discounted. Incubation media routinely contained gentamicin, a wide-spectrum antibiotic with both anti-bacterial and anti-fungal specificities. Incubations were usually short (2 to 6 hours) and were performed at room temperature. Moreover, spectrophotometric analysis of an iris extract-hyaluronate digestion mixture indicated the absence of the Δ -4,5-unsaturated uronides that are characteristic of the oligosaccharide fragments formed by the action of microbial hyaluronidases on hyaluronic acid or chondroitin sulfate substrates (Linker, 1966). It can be concluded that the endohexosaminidase activity of the iris extracts is due to an endogenous iris tissue hyaluronidase.

The narrow pH optimum of the iris hyaluronidase at pH 5.0-5.5 suggests a lysosomal origin for the enzyme. It is widely believed that the intracellular lysosomes are the principal sites of GAG degradation in a majority of tissues (Dorfman *et al.*, 1972; Buddecke and Kresse, 1974). Aronson and Davidson (1968) have demonstrated the uptake and breakdown of sulfated GAGs within liver cell lysosomes. The majority of known vertebrate tissue hyaluronidases, with the notable exception of testicular hyaluronidase, appear to be of lysosomal origin (Meyer, 1971). Unlike the iris

tissue hyaluronidase, however, these hyaluronidases generally have lower pH optima in the region of pH 3.5-4.1 and exhibit little activity above pH 4.5 (Bollet *et al.*, 1963; Aronson and Davidson, 1967a, b; Vaes, 1967; Tan and Bowness, 1968; Cashman *et al.*, 1969; Margolis *et al.*, 1972; Polansky *et al.*, 1974; Orkin and Toole, 1980; Sampaio and Dietrich, 1981). Exceptions are a lysosomal hyaluronidase from the rat kidney that exhibits optimum activity from pH 5.0 to pH 6.1 (Kawamura, 1970), and a hyaluronidase from embryonic mouse salivary gland that has pH optima at pH 5.0 and pH 6.5 (Banerjee and Bernfield, 1979). Testicular hyaluronidase exhibits a broad pH optimum between pH 4.0 and pH 6.0 (Meyer, 1971; Barrett and Heath, 1977). Although secretion of lysosomal enzymes may occur in some cell types (Davies and Allison, 1976), it is unlikely that the iris tissue hyaluronidase functions extracellularly since the enzyme has negligible activity above pH 6.5.

A prominent feature of the newt iris hyaluronidase is its exceptionally high activity in the iris tissue. Crude iris extracts released N-acetylhexosamine from hyaluronate substrate at a rate of approximately 2.3 μ mole acetylhexosamine/h/mg protein at room temperature and pH 3.7. This is almost as high as the level of testicular hyaluronidase activity in crude bovine seminal extracts (2.9 μ mol acetylhexosamine/h/mg protein) as reported by Morton (1976). By contrast the endogenous hyaluronidase activities reported in the regenerating newt limb and several embryonic tissues

are 6 to 100 times lower than the specific activity of the newt iris hyaluronidase (Toole and Gross, 1971; Toole and Trelstad, 1971; Toole, 1972; Orkin and Toole, 1978; Sampaio and Dietrich, 1981). Given the level of hyaluronidase activity in the newt iris tissue as measured in the present study (approximately 10 μ g acetylhexosamine released from hyaluronate per hour per milligram tissue dry weight) and assuming that the concentration of GAGs in the newt iris is similar to that of the rabbit iris as determined by Knepper *et al.* (1981) (approximately 3.5 μ g GAG per milligram tissue dry weight), it can be estimated that the total content of hyaluronidase-sensitive GAG in the newt iris could theoretically be digested to tetrasaccharide fragments within less than one hour. This suggests that the newt iris maintains an excess of hyaluronidase over that required for the normal turnover of GAGs in the tissue matrices.

The present studies have demonstrated that hyaluronate and chondroitin 6-sulfate serve as substrates for the iris hyaluronidase. From the substrate specificity of other tissue hyaluronidases (Meyer, 1971), it is expected that chondroitin 4-sulfate would also be susceptible to the iris enzyme, although this could not be confirmed in the present study because reducing acetylhexosamine residues bearing C-4 sulfate groups do not form coloured reaction products in the assay method of Reissig *et al.* (1955) (Mathews and Inouye, 1961). It is also likely that the iris hyaluronidase

dase would cleave copolymeric dermatan sulfate at the sites of glucuronate residues, but this could not be tested by the method of Reissig *et al.* (1955) for the same reason as above.

When the hyaluronidase activities of extracts of normal irises and irises at three stages of regeneration (10, 15, and 30 days) were compared, there were no marked differences in their rates of hydrolysis of exogenous hyaluronate substrate in the *in vitro* assay. This may indicate that the rate of hyaluronate degradation in the iris remains nearly constant throughout lens regeneration. Accordingly, the rise and subsequent decline in the rate of hyaluronate production in the iris following lentectomy (RESULTS--section II-A) would be due to fluctuations in the rate of hyaluronate polymer assembly rather than due to altered rates of polysaccharide catabolism. This would be in contrast to the situation encountered in several developmental systems where changes in the rates of hyaluronate synthesis are inversely correlated with the levels of endogenous hyaluronidase activity within tissues (Toole and Gross, 1971; Toole, 1972; Toole and Trelstad, 1971; Orkin and Toole, 1978; Belsky and Toole, 1983).

On the other hand, it is by no means certain that the level of enzyme activity in the iris tissue extract *in vitro* provides an accurate index of the rate of hyaluronate degradation in the intact iris *in vivo*. Glaser and Conrad (1979) have demonstrated that while chick embryo chondro-

cytes in culture do not degrade chondroitin sulfate, extracts of these chondrocytes exhibit high levels of hyaluronidase and exoglycosidase activities towards exogenous chondroitin sulfate substrate *in vitro*. Accordingly, it is conceivable that the rate of hyaluronate catabolism in the intact newt iris may be regulated by metabolic mechanisms that depend on cellular integrity. For example, the degradation of glycosaminoglycans by intact cultured fibroblasts relies on receptor-mediated endocytosis of the substrate polymers and transport of the GAG to the lysosomal site of intracellular digestion (Kresse *et al.*, 1975b; Prinz *et al.*, 1978).

It is interesting to note that the existence of hyaluronidase activities in the iris and other ocular tissues has been the subject of some controversy for many years. In early papers on the biochemistry of the ocular fluids and the properties of bacterial hyaluronidases, Meyer and Palmer (1936) and Meyer *et al.* (1936, 1937) made brief references to the presence of hyaluronidase activity in homogenates of rabbit iris and ciliary body. These were the first reports of a hyaluronidase of animal origin in a tissue other than the mammalian testis. Chain and Duthie (1940) were unable to confirm the presence of the iris hyaluronidase and dismissed the findings of Meyer and his colleagues as the result of bacterial contamination of the tissue extracts. Later, Meyer (1947) reported the presence of hyaluronidase activity in bovine aqueous humour and

Brunish *et al.* (1954) described hyaluronidase activity in the bovine vitreous body. However other authors failed to detect hyaluronidase action in aqueous humour (Barany and Woodin, 1955; Mayer *et al.*, 1956; Langley and McCulloch, 1958) or in iris, ciliary body, or cornea (Mayer *et al.*, 1956). With the aid of a highly sensitive dye-binding assay for hyaluronidase activity, Hayasaka and co-workers have recently established the presence of lysosomal hyaluronidases in rabbit and bovine irises as well as in ciliary body, retina, and corneal tissues (Hayasaka and Sears, 1978a, b; Hayasaka *et al.*, 1980, 1981). Kasavina and Chesnokova (1973) have also reported hyaluronidase activity in ocular tissues. The rabbit iris hyaluronidase described by Hayasaka and Sears (1978a) differs from the newt iris hyaluronidase in that the former has optimum activity at pH 3.8 and no activity above pH 5.0 while the newt enzyme has optimum activity at pH 5.0-5.5. In addition, whereas the level of hyaluronidase activity is extremely high in the newt iris, the activity in the rabbit iris appears to be one to two orders of magnitude lower and requires ultra-sensitive assay methods for its detection (Hayasaka and Sears, 1978a).

G) *Distribution of GAG Synthetic Activity
in Iris and Lens Tissues:*

Results from the present studies allow some inferences to be made regarding the predominant sites of GAG synthesis

during lens regeneration. Comparisons of the relative amounts of labelled precursor uptake into GAGs in dorsal iris, ventral iris, and lens tissues indicate that the bulk of GAG synthesis occurs in iris tissue proper (RESULTS--sections I-C, II-A, B). Mature lenses of unoperated or sham-lentectomized eyes incorporated only negligible amounts of $^{35}\text{SO}_4$ and ^3H -glucosamine precursors into CPC-precipitable GAGs. The uptake of $^{35}\text{SO}_4$ label into GAGs was also very low in the tissue of newly regenerated lens rudiments at 30 days after lentectomy (RESULTS--section I-C). These results suggest that GAGs are not major synthetic products of differentiated or developing lens tissues in the newt. This is consistent with reports that only small amounts of polysaccharide are present in the vertebrate lens (Permutt and Johnson, 1953). GAG is a minor component of the lens capsule (Laurent *et al.*, 1978) but is absent from the bulk of lens tissue (Johnson *et al.*, 1982).

Within the normal eye, $^{35}\text{SO}_4$ precursor uptake into sulfated GAGs was nearly equivalent in the dorsal and ventral halves of the iris (RESULTS--section I-C). The rates of $^{35}\text{SO}_4$ labelling remained similar in the dorsal and ventral iris sectors throughout lens regeneration, in spite of the overall increase in sulfated GAG synthesis within both tissues. Clearly, the elevated production of sulfated GAGs that followed lentectomy was not restricted to the lens-forming region of the iris. It therefore seems unlikely that the rise in sulfated GAG synthesis is intimately

associated with the process of cell-type conversion, which is limited to a topographically defined population of depigmented iris epithelial cells at the dorsal pupillary margin. Since the production of sulfated GAGs is elevated throughout the iris, it must involve the large non-lens-forming regions of the tissue where activated iris epithelial cells undergo only partial dedifferentiation and subsequently redifferentiate into pigmented cells along the pathway of retrieval. It is relevant that sulfated GAG synthesis is a normal feature of the newt iris tissue metabolism. Lentectomy enhances the total amount of sulfated GAG synthesis in the tissue but does not alter the pattern of chondroitin sulfate, dermatan sulfate, and heparin/heparan sulfate production that is characteristic of the differentiated iris tissue (RESULTS--section I-B).

In contrast to the situation with the sulfated GAGs, the synthesis of hyaluronate in the iris appears to be under some level of topographical control. Prior to lentectomy, both dorsal and ventral regions of the iris exhibited low rates of ^3H -glucosamine uptake into hyaluronate (RESULTS--section II-A). After lens removal, however, the labelling of hyaluronate was amplified several-fold in the dorsal iris but increased comparatively little in the ventral sector. Hyaluronate synthesis was maximal from days 10 to 15 of regeneration, corresponding to the period of most pronounced dedifferentiation within the dorsal iris epithelial cell population. The restriction of this high

level of synthetic activity to the lens-forming region of the iris at the time of epithelial cell dedifferentiation suggests that the elevated production of hyaluronate may be part of the sequence of biochemical events that prepares the population of dorsal iris epithelial cells to enter the pathway of cell-type conversion.

In an effort to more specifically define the sites of GAG synthesis and deposition in the iris tissue, a preliminary autoradiographic survey was made of the locations of $^{35}\text{SO}_4$ label uptake in histological sections of normal newt eyes and eyes at an advanced stage (80 days) of lens regeneration. The general utility of $^{35}\text{SO}_4$ as a precursor in autoradiographic studies of GAG metabolism has been well established (Dzieviatkowski, 1958).

The distributions of accumulated ^{35}S label were qualitatively similar in autoradiographs of normal and 80-day regenerate eyes (RESULTS--section III). Both dorsal and ventral regions of the iris tissue were heavily labelled with the radioactive precursor. This distribution is consistent with the earlier biochemical analyses that indicated that the rates of $^{35}\text{SO}_4$ label uptake into CPC-precipitable GAGs were nearly equivalent in the dorsal and ventral iris sectors. Within each half of the iris, the ^{35}S label was associated with both the pigmented iris epithelial cell layers and the iris stroma. Accordingly, the production of sulfated GAGs may involve the synthetic activities of both the pigmented iris epithelial cells and

one or more of the stromal cell populations (fibroblasts, mast cells, iridophores, etc.). This suggestion gains support from the demonstration of chondroitin sulfate, heparan sulfate, and hyaluronate synthesis by cultured mammalian iris melanocytes (Sato *et al.*, 1974; Banks *et al.*, 1976; Bhavanandan, 1981) and from the histochemical demonstration of the presence of sulfated GAGs within the stromal matrix of human iris tissue (Sames and Rohen, 1978).

Unfortunately, interpretations based on the present autoradiographic evidence cannot be accepted without reservation. There was a major discrepancy between the autoradiographic profile of $^{35}\text{SO}_4$ distribution in the eye and the earlier measurements of GAG-bound label in isolated iris and lens tissues. In autoradiographs there were very high densities of ^{35}S label in both mature and 80-day regenerate lenses, despite the fact that biochemical analysis consistently demonstrated only negligible uptake of $^{35}\text{SO}_4$ precursor into CPC-precipitable GAGs within normal and regenerating lens tissues. Investigations by Dohlman (1957) may be relevant to this paradoxical situation. In a study of $^{35}\text{SO}_4$ uptake in the rabbit eye, Dohlman determined that the vast majority of ^{35}S label associated with lens tissue was present in low molecular weight dialyzable form; precursor uptake into the ester-sulfate groups of macromolecules such as GAGs was negligible in the lens. It was also found that $^{35}\text{SO}_4$ label readily adsorbed to lens tissue and was not effectively removed during the fixation and dehydration

procedures that preceded autoradiography. In contrast, in other ocular tissues, including the iris, the majority of ^{35}S label was associated with the ester sulfates of macromolecules although there was some additional non-specific uptake into non-sulfate sulfur containing macromolecules.

It is clear that it cannot be assumed that all the $^{35}\text{SO}_4$ label in autoradiographs of ocular tissues is associated with the ester sulfate groups of macromolecules. The potential for non-specific label uptake is apparently highest in the lens, but to a lesser extent may also affect the pattern of label distribution in other ocular tissues including the iris (Dohlman, 1957). Moreover, not all the ester sulfate groups of macromolecules are necessarily associated with GAGs, since sulfated carbohydrate moieties are also present in some glycoproteins and glycolipids (De Meio, 1967; Balasubramanian and Bachhawat, 1970; Farooqui, 1978).

No empirical efforts were made to identify the nature of the sulfate label associated with the new lens tissue. It may represent adsorption of inorganic $^{35}\text{SO}_4$ precursor to the lens during the *in vivo* labelling period as suggested by the study of Dohlman (1957). Another possibility is that $^{35}\text{SO}_4$ label may be incorporated into sulfated-glutathione conjugates in the lens. S-sulphoglutathione has been demonstrated in mammalian lens tissue (Waley, 1959) and derives its sulfate groups from inorganic sulfate precursor *via* the 3'-phosphoadenosine 5'-phosphosulfate intermediate (De Meio, 1967).

II. Possible Roles of GAGs in the Iris during Lens Regeneration

The functional significance of enhanced hyaluronate and sulfated GAG synthesis to the cytological events within the iris during lens regeneration is not yet apparent. Nevertheless, information regarding the physiological roles of GAGs in other biological systems provides some ground for speculation on the nature of GAG involvement in the processes of iris epithelial cell dedifferentiation, redifferentiation, and cell-type conversion.

Evidence from the present study indicates that the iris tissue of the newt produces heparin/heparan sulfate, chondroitin sulfate, dermatan sulfate, and hyaluronate polymers as part of its normal metabolism. In other tissue systems these GAGs are most commonly components of cell-surface coats (Kraemer 1971a, b; Dietrich and DeOca, 1978; McBride and Bard, 1979; Underhill and Toole, 1982; Oldberg *et al.*, 1981; Kjellen *et al.*, 1980, 1981), intercellular matrices (Dietrich *et al.*, 1977; Hedman *et al.*, 1982) and epithelial basement membranes (Cohn *et al.*, 1977; Gordon and Bernfield, 1980; Kanwar and Farquhar, 1979; Hassell *et al.*, 1980). By analogy with these systems, it is presumed that the GAGs of the newt iris contribute to the cell-surface structures, extracellular ground substance, and epithelial basement membranes of the tissue. The basal level of GAG production in the untraumatized newt iris

would likely be involved in the maintenance of these structures and would thereby contribute to the preservation of normal tissue integrity.

The cellular origins of the GAG synthetic activity in the normal newt iris have not yet been defined. The autoradiographic profile of $^{35}\text{SO}_4$ uptake in the iris suggests the possibility that sulfated GAG synthesis occurs in both the stromal and epithelial compartments of the iris tissue. However, as discussed earlier, the autoradiographic distribution of $^{35}\text{SO}_4$ precursor may not provide a specific marker of the localization of sulfated GAG polymers in the iris. There is the additional complication that labelled GAGs synthesized in one part of the tissue might be translocated and then deposited at another site in the iris.

Since the pigmented iris epithelial cells constitute the largest cell population in the iris, it is likely that they are largely responsible for the production of GAGs in the tissue. The capacity for GAG synthesis by pigmented iris epithelial cells has been demonstrated in the cultured iris melanocytes of both mice and humans (Sato *et al.*, 1974; Banks *et al.*, 1976; Bhavanandan, 1981). The presence of GAGs at the cell-surfaces of the newt iris epithelial cells is suggested by the demonstration that chondroitinase and testicular hyaluronidase treatments decrease the electrophoretic mobilities of dissociated newt iris epithelial cells (Zalik and Scott, 1973; Zalik *et al.*, 1976). In histological sections, the basement membrane associated with

the inner lamina of newt iris epithelial cells stains with the cationic dye, Alcian Blue (Kulyk and Zalik, unpublished observations). Alcian Blue staining by the method of Mowry (1963) is a standard histochemical procedure for demonstrating GAGs in tissues (Pearse, 1968). By the immunofluorescence technique, the newt iris epithelial basement membrane was also found to contain fibronectin (Kulyk, unpublished observations). Fibronectin is an extracellular glycoprotein that is frequently co-distributed with heparan sulfate proteoglycans in the pericellular matrices of cultured mammalian cells (Hayman *et al.*, 1982; Hedman *et al.*, 1982) and in the basement membranes of tissues (Timpl and Martin, 1982). In light of this evidence, it may be expected that the pigmented epithelial cells of the normal newt iris are active in the synthesis of GAG polymers and that these GAGs contribute to the glycocalyx, basement membrane, and intercellular matrix structures of the iris epithelium.

The fibroblasts in the connective tissue stroma of the newt iris are another likely source of GAG synthetic activity. The stromal matrix of the newt iris stains with Alcian Blue dye and demonstrates positive immunofluorescence for fibronectin (Kulyk and Zalik, unpublished observations). Sames and Rohen (1978) have demonstrated the presence of both sulfated GAGs and hyaluronate in the human iris stroma by Alcian Blue staining and selective enzyme treatments. It is possible, however, that the rate of GAG synthesis in the newt iris stroma may be considerably lower

than in the pigmented epithelium, since the population of fibroblasts in the iris is small in comparison with the pigmented epithelial cell population. Also, the turnover of GAGs in connective tissue matrices is frequently slower than in epithelial matrices (see DISCUSSION--section I-B).

Since the vast majority of sulfated GAGs in vertebrate tissues are associated with protein as proteoglycan complexes (INTRODUCTION--section III-B), it is reasonable to presume that the GAGs of the newt iris are also proteoglycan constituents in their native forms. To what extent the dermatan sulfate, chondroitin sulfate, and heparin/heparan sulfate polymers are linked to common or different core proteins has not been investigated. As suggested earlier, the co-distribution of the different sulfated GAG types on common core proteins could provide a simple mechanism whereby the rates of synthesis of the various sulfated GAG species could be coordinately amplified as is observed in the iris during lens regeneration. Examples from mammalian systems, however, suggest that heparan sulfate and chondroitin sulfate polymers usually reside in different proteoglycan monomers (Bland *et al.*, 1982; Kanwar *et al.*, 1981). In contrast to the sulfated GAGs, hyaluronate polymers are usually free polysaccharides *in situ* (INTRODUCTION--section III-A) and would presumably exist as free GAG chains in the newt iris tissue as well.

Elevated rates of radioactive precursor uptake following lentectomy indicate that lens regeneration is accom-

panied by enhanced production of both sulfated GAGs and hyaluronate in the iris tissue. The gross spatial distribution of this augmented synthetic activity within the tissue as well as the timing of maximum hyaluronate and sulfated GAG production may provide some clues as to physiological significance of elevated GAG synthesis to the regeneration process. Evidence that the synthesis of sulfated GAGs is increased to similar extents in the dorsal (lens-forming) and ventral (non-lens-forming) regions of the iris argues against an intimate involvement in the topographically restricted process of iris epithelial cell-type conversion. It is more likely that the enhanced sulfated GAG production is related to the metabolic activities of the large numbers of iris epithelial cells in both the dorsal and ventral regions of the iris that undergo only partial dedifferentiation following lentectomy and then revert to their original phenotype as melanocytes. This is supported by the observation that the pattern of chondroitin sulfate, dermatan sulfate, and heparin/heparan sulfate production in the regeneration-activated iris is similar to the pattern in the normal iris despite the overall increase in sulfated GAG synthesis after lentectomy. This characteristic pattern of sulfated GAG production may be an expression of the iris epithelial melanocyte phenotype and would consequently be lost by the population of epithelial cells that undergo complete dedifferentiation and embark on the pathway of conversion into lens fiber cells. In this

context, it is relevant that the lens rudiments of 30-day regenerate eyes have a very low level of sulfated GAG synthesis.

While maximum sulfated GAG synthesis appears to occur around 15 days after lentectomy, it is noteworthy that a high level of sulfated GAG production is maintained throughout the time-course of lens regeneration (*i.e.* from day 5 to at least day 30 after lentectomy). The processes of melanocyte dedifferentiation and retrieval do not proceed synchronously in the iris epithelial cell population and therefore are similarly spread over a large part of the regeneration period. DNA replication and mitosis are first observed in the iris epithelium at 4 to 5 days after lentectomy (T. Yamada and Roesel, 1969, 1971), but the entrance of some pigmented epithelial cells into the cell cycle is delayed for 2 to 3 days (T. Yamada, 1977). In addition, the activated epithelial cells outside the dorsal-marginal region of the iris attain varying degrees of dedifferentiation and depigmentation prior to withdrawing from the cell cycle. Accordingly, the resynthesis of melanosomes and other metabolic processes of melanocyte retrieval probably also proceed asynchronously in the cell population. Since sulfated GAG production appears to be an expression of the epithelial melanocyte phenotype, the peak of sulfated GAG synthesis at day 15 of regeneration may reflect the point at which the largest number of iris epithelial cells are undergoing the growth processes associated

with melanocyte redifferentiation. These growth processes would presumably include production of the cell-surface materials and basement membrane structures characteristic of the normal iris.

In contrast to the situation with the sulfated GAGs, the elevated production of hyaluronate following lentectomy appears to be topographically regulated. The restriction of maximal hyaluronate synthesis to the dorsal region of the iris during the period from 10 to 15 days after lentectomy suggests an involvement of enhanced hyaluronate production with the dedifferentiation of those iris epithelial cells that embark on the pathway of cell-type conversion. In a variety of other developmental systems there is a correlation between elevated production and accumulation of hyaluronate within tissues and the timing of proliferative and migratory activities in undifferentiated embryonic cell populations (Toole and Trelstad, 1971; Toole, 1972; Pratt *et al.*, 1975; Solursh, 1976; Solursh and Morriss, 1977; Fisher and Solursh, 1977; Orkin and Toole, 1978; Derby, 1978; Pintar, 1978; Belsky and Toole, 1983). Notably, the period of proliferation and migration of dedifferentiated blastemal cells in the regenerating newt limb is characterized by an increased synthesis and tissue-content of hyaluronate (Toole and Gross, 1971). Toole (1973, 1976, 1981) has advanced the hypothesis that hyaluronate-rich extracellular matrices promote cell motility and proliferation and inhibit precocious differentiation. In keeping with this

hypothesis, the increased accumulation of hyaluronate in the dorsal iris following lentectomy may play a role in sustaining the mitotic proliferation of the dedifferentiated iris epithelial cells at the dorsal pupillary margin of the iris. Iris epithelial cells apparently must traverse a critical number of cell cycles in order to differentiate as lens fibers (T. Yamada, 1977; T. Yamada and Beauchamp, 1978). By promoting prolonged cell proliferation, hyaluronate may prevent precocious redifferentiation of the dorsal-marginal iris epithelial cells into melanocytes and thereby encourage their completion of the regenerative program of cell-type conversion. While migratory activity is negligible among the depigmented iris epithelial cells, an elevated hyaluronate content in the intercellular spaces may facilitate infiltration of the tissue by macrophages and mast cells (Setoguti *et al.*, 1963; T. Yamada and Dumont, 1972).

Growth and maturation of the lens vesicle at the dorsal pupillary margin is apparently accompanied by a relative decline in the rate of hyaluronate production in the dorsal iris. Toole and his associates have demonstrated that in several developmental systems the onset of cytodifferentiation coincides with a reduction in the synthesis and absolute amount of hyaluronate in tissues (Toole and Trelstad, 1971; Toole and Gross, 1971; Toole, 1972; Orkin and Toole, 1978; Belsky and Toole, 1983). In each of these cases, the decline in hyaluronate production and accumula-

tion was correlated with an increase in the activity of an endogenous hyaluronidase in the tissue. The present study has established the presence of a potent hyaluronidase in the newt iris. However, comparisons of hyaluronidase activities in extracts of whole irises at different stages of regeneration have revealed no prominent stage-dependent variation. As suggested earlier, this does not preclude the possibility that the decline in the net level of hyaluronate synthesis in the dorsal iris between days 15 and 20 of regeneration may be mediated by an increase in the rate of hyaluronate catabolism in the tissue. The rate of GAG polymer degradation *in situ* may be regulated by a metabolic process that limits the availability of substrate to the glycosaminoglycan-degrading enzymes, rather than by the concentration of enzymes in the tissue. The exceptionally high hyaluronidase activity in the iris suggests that the enzyme is always present in excess of the amount required to completely degrade the hyaluronate and chondroitin sulfate constituents of the iris.

Since the acidic pH optimum of the iris hyaluronidase suggests a lysosomal site of action, a system is probably required to transport the extracellular GAGs of the iris to their intracellular locus of degradation. Studies on cultured fibroblasts indicate that cells internalize extracellular GAGs and proteoglycans by receptor-mediated endocytosis (Kresse *et al.*, 1975b; Prinz *et al.*, 1978). Using systems of receptor-mediated endocytosis, the iris epithelial

cells could theoretically modify their rates of GAG catabolism *in situ* by regulating the availability of specific cell-surface receptors or the rates of formation and transport of endocytotic vesicles. The presence of hyaluronate-specific binding sites and heparan sulfate-specific receptors has been demonstrated in other cell types (Underhill and Toole, 1979, 1980; Kjellen *et al.*, 1980, 1981; Kraemer, 1977). Receptor-mediated endocytosis could provide a mechanism whereby the catabolism of different GAG species in the newt iris could be differentially regulated.

In the foregoing discussion it has been largely assumed that the dedifferentiating and redifferentiating iris epithelial cells themselves are the source of enhanced hyaluronate and sulfated GAG production during lens regeneration. This is reasonable as the iris epithelial cells are the most metabolically dynamic cell population in the iris during lens regeneration. Nevertheless, the possibility remains that GAG synthesis could also be enhanced in the connective tissue stroma of the iris that overlies the outer layer of pigmented epithelium. Biochemical changes in the iris stroma could be relevant to the cytological events in the adjacent epithelial cells. Lopashov (1977) and T. Yamada (1977) have suggested that the stroma may suppress cell-type conversion in the underlying pigment cells. In iris epithelial cell cultures, the presence of stroma or stromal fibroblasts promotes the attachment, spreading, migration, and shedding activities of pigment

cells (T. Yamada, 1977; also Zalik and Dimitrov, unpublished observations). This suggests that stromal cells or their synthetic products may influence the behaviour of iris epithelial cells *in situ*.

Another possibility is that increased GAG synthesis following lentectomy may be associated with the metabolism of macrophages and mast cells that invade the iris epithelium during lens regeneration (Setoguti *et al.*, 1963; T. Yamada and Dumont, 1972). Mast cells, in particular, are known to contain heparin and other sulfated GAGs (Jaques, 1982; Bland *et al.*, 1982). However it is doubtful that mast cells or macrophages contribute substantially to the elevated production of sulfated GAGs observed during lens regeneration. The pattern of heparin/heparan sulfate, chondroitin sulfate and dermatan sulfate production in the regenerating iris is not altered from that of the normal iris in which macrophage and mast cell numbers are extremely low (T. Yamada and Dumont, 1972). The predominance of elevated hyaluronate production in the dorsal region of the iris also argues against an intimate association with the synthetic activities of invading macrophages or mast cells, as these cells infiltrate both dorsal and ventral areas of the iris in similar numbers (T. Yamada and Dumont, 1972).

The source of hyaluronidase activity in the iris is almost certainly the pigmented iris epithelial cells. The stromal fibroblast population is probably too small to account for the exceptionally high level of enzyme activity

in the iris tissue. Although some macrophages contain hyaluronidase (Goggins *et al.*, 1968), the level of iris hyaluronidase activity remains similar before and after lenticectomy, despite the fact that there are few macrophages in the normal iris and large numbers in the regeneration-activated tissue (T. Yamada and Dumont, 1972).

III.A Model of GAG Involvement in Iris Epithelial
Cell Dedifferentiation, Retrieval,
and Cell-Type Conversion

The preceding discussion suggests a tentative model for the involvement of GAGs in the cytological events of lens regeneration. This model is consistent with the interpretations of results from the present study and with some relevant observations from previous work in the fields of lens regeneration and extracellular matrix biology. This model is only novel in respect to the functions of sulfated GAGs and hyaluronate in the process of lens regeneration and, as such, incorporates hypotheses advanced previously by other authors. While many aspects of the present model are admittedly highly speculative, it suggests a number of testable hypotheses that could form the basis of future research on the roles of GAGs and other matrix components in the process of newt lens regeneration.

The model proposes that the mitotically quiescent pigmented iris epithelial cells of the normal iris have a continuous basal rate of synthesis of sulfated glycosaminoglycans (heparan sulfate, chondroitin sulfate, and dermatan sulfate) and a very low level of hyaluronate synthesis. In keeping with the general pattern in vertebrate tissues (INTRODUCTION--section II-B), the sulfated GAG polymers are assembled as side-chains of larger proteoglycan complexes. Synthesis occurs in the rough endoplasmic reticulum and

Golgi apparatus of the iris epithelial cells and the assembled proteoglycans are transferred to the cell surface *via* an exocytotic system of membrane bound vesicles. Synthesis of hyaluronate is also initiated on intracellular membranes (although probably in the absence of a primer protein; see INTRODUCTION--section II-B) and the hyaluronate is secreted from the iris epithelial cells as free GAG chains.

It is proposed that a large proportion of the sulfated proteoglycans are assimilated into the surface coats of the iris melanocytes, either as integral membrane proteins (analogous to the lipid-bound heparan sulfate proteoglycans demonstrated by Kjellen *et al.*, 1981, and Norling *et al.*, 1981) or in association with specific binding sites on the cell surface (similar to the heparan sulfate proteoglycan receptors demonstrated by Kraemer, 1977; Kjellen *et al.*, 1980; Norling *et al.*, 1981). These surface localized proteoglycans would presumably contribute to the intercellular adhesive complexes that bind neighbouring cells in the melanocyte population and maintain their normal compact epithelial organization. A proportion of the sulfated proteoglycans produced by the iris epithelial cells would alternatively be secreted and serve as structural elements in the basement membranes surrounding the iris epithelium. Recent studies suggest that heparan sulfate proteoglycans may be universal components of basement membranes (Gordon and Bernfield, 1977; Kanwar and Farquhar, 1979; Hassell *et al.*, 1980). It is possible that some of the proteoglycans

secreted by the iris epithelial cells might also contribute to the extracellular stromal matrix adjacent to the anterior border of the pigmented iris epithelium.

The small amounts of hyaluronate produced by the iris epithelial cells might associate with cell-surface binding sites (analogous to the hyaluronate receptors of cultured fibroblasts demonstrated by Underhill and Toole, 1979, 1980) or might serve as nucleation sites for the aggregation of sulfated proteoglycans in the intercellular matrices (as in the cartilage matrix proteoglycans; see INTRODUCTION--section II-B).

As is the case in many other tissues, the synthesis of GAG polymers in the normal iris is presumed to be restricted at a low level by limited availability of the core proteins that serve as initiation sites for polysaccharide chain assembly. The GAG composition of the iris epithelium and its associated matrix structures is maintained in a steady state by a continuous basal turnover of proteoglycans and hyaluronate. The catabolism is regulated by the rate of receptor-mediated endocytosis of cell-surface and extracellular matrix GAGs. Digestion occurs intracellularly in lysosomes and is initiated by protease action (to degrade core protein) and by hyaluronidase action (against GAG polymers). Digestion is completed by the stepwise action of exoglycosidases, including β -N-acetylhexosaminidase (Idoyaga-Vargas and Yamada, 1974).

As proposed previously by other authors (see INTRODUC-

TION--section II-F), removal of the lens of the eye is presumed to expose the pigmented iris epithelial cells to a diffusible growth factor released from the retina. The retinal factor molecules may bind to specific receptors on the iris epithelial cell surfaces (as proposed by Cuny, 1982) and initiate a sequence of metabolic changes in the melanocytes. Cytoplasmic cyclic AMP levels are known to decline sharply between days 1 and 2 after lentectomy and then rise to levels above those in the normal iris (Thorpe *et al.*, 1974; Velazquez and Ortiz, 1980). The altered cyclic AMP levels may stimulate the observed increase in ribosomal RNA synthesis in the iris epithelial cells (T. Yamada and Karasaki, 1963; Reese *et al.*, 1969; Thorpe *et al.*, 1974; see INTRODUCTION--section II-E) and the resulting increase in the density of ribosomes in the melanocyte cytoplasm (Eguchi, 1963; Karasaki, 1964). The latter is probably responsible for the rise in the level of protein synthesis between days 2 and 8 after lentectomy (T. Yamada and Takata, 1963).

The modulation of cyclic AMP levels is also presumed to induce cell-surface alterations in the iris epithelial cells. Electron microscopic studies reveal that the dedifferentiation and depigmentation of iris epithelial cells are accompanied by the formation of numerous microvillar cell processes (Dumont and Yamada, 1972) and by the shedding of negatively charged portions of the cell membrane (T. Yamada, 1977). The loss of negatively charged surface

groups is confirmed by the decline in the electrophoretic mobilities of dissociated iris epithelial cells between days 3 and 10 after lentectomy (Zalik and Scott, 1972). The present model suggests that at least some of the anionic components lost from the pigment cell periphery early in the dedifferentiation process are the surface-associated sulfated proteoglycans and hyaluronate. This is supported by the apparent disappearance from the iris epithelial cell surface of negatively charged components that are sensitive to chondroitinase ABC and testicular hyaluronidase treatments during the period between 3 and 5 days after lentectomy (Zalik and Scott, 1973; Zalik *et al.*, 1976).

The shedding of membrane by the dedifferentiating iris epithelial cells results in a loss of other cell-surface components in addition to GAGs. Studies by Zalik and Scott (1973) and Zalik *et al.* (1976) suggest that there is a disappearance of glycoproteins bearing terminal sialic acid residues between days 7 and 10 after lentectomy. The present model proposes that the membrane shedding also depletes the cell surface of specific proteoglycan and hyaluronate binding sites.

A desquamation of cell-surface heparan sulfate precedes mitosis in some cultured cells (Kraemer and Tobey, 1972) and cell transformation *in vitro* is often accompanied by a reduction in cell-surface heparan sulfate (Chiarugi *et al.*, 1974, 1981). On the basis of these correlations, Chiarugi and Vannucchi (1976) have suggested a model in

which a high density of polyanionic heparan sulfate at the cell surface inhibits mitosis and cell growth (*i.e.* maintains cells in a resting state or G_0). In keeping with this model, it is proposed that a loss of sulfated proteoglycans from the peripheries of the newt iris epithelial cells between days 3 and 5 after lentectomy results in a decline in their negative cell-surface charge. This reduced surface potential releases the melanocytes from their normal inhibition of DNA replication and mitosis. The pigment cell population is thereby activated into proliferation.

The net increase in the level of sulfated GAG production that is observed in the iris by five days after lentectomy is attributed to two processes. Due to the increased number of ribosomal sites of protein synthesis in the cytoplasm of the depigmenting iris epithelial cells (Eguchi, 1963; Karasaki, 1964) there is an increase in the rate of translation of mRNA sequences coding for proteoglycan core proteins. The increased availability of the core proteins that serve as initiation sites for GAG polymer assembly stimulates an elevated rate of GAG synthesis in the iris. The enhanced production of core proteins may account in part for the increased rate of protein synthesis reported in both dorsal and ventral regions of the iris epithelium between days 2 and 16 after lentectomy (T. Yamada and Takata, 1963). The increased glutamine synthetase activity in the iris during dedifferentiation (Thorpe *et al.*, 1974)

may relate to the biosynthetic requirement for glutamine in the transfer of amino groups to the hexosamine precursors of the elongating GAG polymers. As suggested above, the shedding of membrane materials from the dedifferentiating iris epithelial cells depletes the cell surface of specific hyaluronate and proteoglycan binding sites. This impairs the system of receptor-mediated endocytosis by which GAGs are internalized and transferred to the lysosomes for degradation. The resulting decrease in the rate of GAG catabolism by the iris epithelial cells augments the already elevated rate of GAG accumulation in the iris tissue.

The reduction in the numbers of proteoglycan and hyaluronate binding sites on the dedifferentiating iris epithelial cells also inhibits the retention of newly synthesized GAGs at the cell surface. The newly formed hyaluronate and proteoglycans are instead discharged freely into the intercellular spaces. This accounts for the observation that the negative surface potential of the dedifferentiating pigment cells remains depressed until day 15 of regeneration (Zalik and Scott, 1972) in spite of their increased GAG synthetic activity. The initial loss of cell-surface GAGs and GAG binding sites is presumed to weaken the intercellular adhesion of the iris epithelial cells. The secretion of large quantities of proteoglycans between neighbouring cells may explain the expansion of intercellular spaces described by Dumont and Yamada (1972). These effects contribute to a disruption of the normal compact

iris epithelial morphology and may reduce the intimacy of the association of iris epithelial cells with their basement membrane. Concomitantly, invading macrophages digest portions of the basement membrane and engulf melanosomes extruded from the depigmenting iris epithelial cells (Dumont and Yamada, 1972; T. Yamada and Dumont, 1972).

Under some form of topographical control, the production of hyaluronate is greatly elevated at the dorsal margin of the iris and is only slightly elevated in the ventral iris. This may be due to an intrinsic difference in the capacity for hyaluronate synthesis by dorsal and ventral iris epithelial cell populations, but is more likely a response to some as yet undefined positional cues. The hyaluronate secreted in the dorsal region of the iris, by virtue of its high water-binding capacity and strong osmotic potential (Ogston, 1970; Comper and Laurent, 1978; Toole, 1981), promotes swelling of the intercellular spaces and contributes to the enlargement of the space between the anterior and posterior layers of the dorsal iris epithelium. In keeping with the putative role of hyaluronate as a promoter of cell proliferation and an inhibitor of differentiation (Toole, 1973, 1976, 1981; Chiarugi *et al.*, 1979), the high concentration of hyaluronate at the dorsal iris margin during the period of most active iris epithelial cell dedifferentiation and depigmentation may help to maintain the cells in a proliferative state and prevent them from precociously withdrawing from the cell cycle and

reverting to their original melanocyte morphology. By the time a critical number of cell cycles have been traversed by the depigmented cells at the dorsal iris margin (approximately day 15 as proposed by T. Yamada, 1977), these cells have ceased to produce sulfated GAGs in the characteristic pattern of the pigmented melanocyte. Now committed to the pathway of cell-type conversion, their rate of hyaluronate production declines. A gradual reduction in the hyaluronate content of the dorsal iris may be promoted by recovery of the surface receptors involved in hyaluronate endocytosis, allowing the subsequent digestion of hyaluronate by hyaluronidase in the intracellular lysosomes. The removal of hyaluronate from the intercellular matrices allows the dedifferentiated iris epithelial cells to redifferentiate as lens fibers.

In the remainder of the iris (*i.e.* non-lens-forming regions) the partially depigmented iris epithelial cells gradually regenerate their cell-surface receptors for sulfated proteoglycans and hyaluronate and re-establish their system of endocytosis-mediated GAG catabolism. The re-appearance of proteoglycan receptors promotes binding of secreted sulfated GAGs to the cell surface and contributes to a gradual recovery of the negative surface charge of the iris epithelial cells (Zalik and Scott, 1972; Zalik *et al.*, 1976). In accordance with the model of Chiarugi and Vannucchi (1976), the increased density of polyanionic GAGs at the cell surface inhibits further mitotic proliferation and

promotes a withdrawal from the cell cycle. Having failed to traverse a critical number of cell cycles, the partially depigmented cells redifferentiate as melanocytes along the pathway of retrieval (T. Yamada, 1977). The level of sulfated GAG synthesis in the postmitotic redifferentiating pigment cells remains transiently elevated due to reconstruction of cell-surface adhesive components and basement membrane structures that were lost in the dedifferentiation process. Synthesis of other matrix components (*e.g.* fibronectin, laminin, Type IV collagen) is probably also elevated at this time. Thereafter, the level of GAG synthesis is expected to gradually return to the basal level characteristic of the non-regenerating iris.

IV. Suggestions for Future Study

A critical assumption in the preceding model is that the iris epithelial cells are the predominant source of increased GAG synthesis in the iris during lens regeneration. As yet this assumption remains in need of experimental verification. A suitable approach to this problem would be to assess the GAG synthetic activity of iris epithelial cells in culture, in the absence of significant numbers of stromal fibroblasts, macrophages, or mast cells. The model would predict that dedifferentiating iris epithelial cells *in vitro* would incorporate labelled precursors into sulfated glycosaminoglycans and secrete GAGs into the culture medium.

Another assumption of the preceding model is that the sulfated GAGs produced in the iris are side-chains of larger proteoglycan complexes in their native state. Procedures for isolation of proteoglycans are now well established but the small amount of tissue in the newt iris presents technical problems. The latter could be partially circumvented by culturing iris tissue fragments for short periods in media containing labelled precursors for core proteins (e.g. ^3H -leucine) and GAG polymers (e.g. $^{35}\text{SO}_4$ or ^{14}C -glucosamine). Isolation could then be conducted in the presence of unlabelled proteoglycan carriers and a variety of fractionation methods could be used to determine to what extent any doubly-labelled macromolecules are sensitive to

protease and polysaccharidase treatments.

The proposed model also presumes the presence of a receptor-mediated system of endocytosis by which iris epithelial cells internalize GAGs and proteoglycans prior to degradation. It would be worthwhile to assess the capacity of cultured iris epithelial cells to internalize and degrade exogenous labelled GAG and proteoglycan substrates. The model suggests, however, that the dedifferentiation of iris epithelial cells under culture conditions might impair their endocytotic activities.

Antibodies against mammalian heparan sulfate proteoglycans and chondroitin sulfate proteoglycans have recently been prepared (Hassell *et al.*, 1980; Oldberg *et al.*, 1981). These may provide sensitive and specific probes for immunofluorescence localization of the GAG components of the iris tissue in histological sections. The possibility of species specificity in these antibodies may, however, introduce technical problems in such an approach.

Other worthwhile subjects of investigation include:

- whether the iris tissue contains a heparan sulfate-degrading endoglycosidase
- whether hyaluronidase activity is identical in dorsal and ventral halves of the iris
- whether the capacity for lentoid formation in iris epithelial cell cultures and lens differentiation in iris organ cultures is affected by addition of exogenous GAGs to the culture medium or by addition of β -D-xylosides

- the extent of the involvement of other matrix components such as laminin, fibronectin, collagen, and cell-surface glycoproteins in the lens regeneration process.

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